PRODUCTION AND OPTIMIZATION OF L-ASPARAGINASE FROM *Pseudomonas aeruginosa* BY STRAIN IMPROVEMENT

Dissertation

Submitted to
The Tamil Nadu DR .M. G. R. Medical University, Chennai
In partial fulfillment for the award of the degree of

MASTER OF PHARMACY
In
PHARMACEUTICAL BIOTECHNOLOGY

By
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DECLARATION

I hereby declare that this thesis work entitled **"PRODUCTION AND OPTIMIZATION OF L-ASPARAGINASE FROM *Pseudomonas aeruginosa* BY STRAIN IMPROVEMENT"** submitted to the Tamil Nadu Dr.M.G.R. Medical University, Chennai was carried out by me in the Department of Pharmaceutical Biotechnology, Ultra College of Pharmacy, Madurai under the valuable and efficient guidance of **Dr.G. RENUGA** and **Prof. A.BABU THANDAPANI**, Department of pharmaceutical Biotechnology, Ultra college of Pharmacy, Madurai during the academic year May 2012- April 2013. I also declare that the matter embodied in it is a genuine work and the same has not formed the basis for the award of any degree, diploma, and associate ship, fellowship of any other university or institution.

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EXAMINERS:
1.

2.

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ACKNOWLEDGEMENT

Say, "Indeed, my prayer, my rites of sacrifice, my living and my dying are for Allah, Lord of the worlds*No partner has He. And this I have been commanded and I am the first [among you] of the Muslims."

I thank the **Almighty Allah** who has blessed me and extended helping hands to achieve this dissertation work successfully.

I explain my extreme sense of gratitude, profound thanks to my Guide **Dr. G. RENUGA** and **Prof.A.BABU THANDAPANI** , in the Department of Pharmaceutical Biotechnology Ultra College of Pharmacy, Madurai for their efficient and valuable guidance, encouragement and abundant help, enthusiastic and inspiring discussion and the timely suggestions which proved for the success of this work.

I would like to express my deepest gratitude to our honorable **Chairman**, **Prof.K.R.ARUMUGAM**, M.Pharm, Ultra College of Pharmacy, Madurai for this valuable and meaningful support to carry out this project.

It is my privilege and honor to extend my profound gratitude and express my indebtedness to our **Dean Dr.C.VIJAYA**, M.Pharm, Ph.D., Ultra College of Pharmacy, Madurai for her constant inspirations, valuable advice, encouragement and innovative ideas throughout the course of the project.

It extend my sincere thanks and respectful regards to our professors**, Mr. K Natarajan, Prof M.Chandran, Dr.K.G.Lalitha, Mr.R.Sathish, Mr.G.Pratheesh, Mr.V.Sivanandham, Mr.T.Regupathi, Mr.N.Sridhar** and **Mr.K.Senthilkumar** for their valuable suggestions throughout my thesis work.

I express my sincere thanks to all my classmates and friends who helped me and blessed in all the way during this work.

I thank to lab assistant Ms.C. Manimegalai, store keeper Mrs.B.Masila, attenders Ms. Poonkodi and Mr.Sonai of Ultra College of Pharmacy, Madurai.

Finally, I present my warm, beloved gratitude with thanks to my Father, Mother, Brothers, Sisters and my dear friend for their blessing and constant encouragement given to me and adored me during the course of my study.

I am very grateful and humble to thank my dearest brother **Kamal Mohamed Osman Ibrahim** for his continuous help and support throughout my study**,** I am very grateful to thank my dear brothers and sisters for their enormous encouragement given to me during my entire study in India.

I whole heartily thank all those who have stretched their helping hands towards me directly or indirectly for successful completion of this thesis work. I extend my sincere thanks to them too.

	TABLES	
Table 1.	**Anticancer drugs (1980)**	**15**
Table 2.	**Anticancer drugs (1999)**	**16**
Table 3	**New approaches in cancer therapy (2000)**	**17**
Table 4.	**List of chemicals used**	**42**
Table 5.	**List of instruments used**	**44**
Table 6.	**Composition of the modified media**	**46**
Table 7.	**Mutation of the strain**	**48**
Table 8.	**Zone diameter of screened strain**	**56**
Table 9.	**Assay of l-asparaginase from crude extracts**	**62**

INTRODUCTION:

1. ENZYME:

Enzymes are biomolecules or proteins materials that catalyze chemical reactions. In enzymatic reactions, the molecules at the beginning of the process are called substrates, and the enzyme converts them into different molecules, called the products. Almost all processes in a biological cell need enzymes to occur at significant rates. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell. [1]

In 1878, German physiologist Wilhelm Kuhner first used the term enzyme, which comes from Greek word “in leaven”, to describe this process. The word enzyme was used later to refer to nonliving substances such as pepsin.

Like all catalysts, enzymes work by lowering the activation energy for a reaction, thus dramatically increasing the rates of the reaction. Most enzyme reaction rates are millions of times faster than those of comparable un-catalyzed reaction. As with all catalysts, enzymes are not consumed by the reactions they catalyze. [2]

Advantages of Enzymes: [2]

Enzymes are a sustainable alternative to the use of dangerous chemicals in industry because of their following advantages:

They are specific, will generate only one product and no other.

Generally operate in aqueous solutions which are cheaper and safer than organic solvent.

Reduce energy consumption by eliminating the need to maintain extreme environments.

Enzymes are both economically and environmentally feasible.

Applications of Enzymes [2]

Uses of enzymes can be classified as:-

(1)Medicinal and Clinical Use:-

Enzymes can be used for Aiding Digestion. Example: Amylases, Proteases and Lipase.

They can also be used as Deworming agents. Example: Papain.

Enzymes act as anti-clotting agents like Fibrinolytic and Thrombolytic. Examples: Urokinase and Streptokinase.

Enzymes can be used as surface disinfectants. Example: Trypsin.

They can also be used in the diagnosis purpose. Example: Glucose oxidase along with peroxidase to detect the level of glucose (colorimetric estimation).

Liver disease	: SGPT, SGOT
Heart attacks	: SGOT
Myocardial Infarction (MI)	: Creatine phosphokinase.

(2)Industrial purpose:-

- Enzymes can be used in the textile industry. Example: Amylase as softening agent for starched clothes.
- They can also be used for Leather purpose. Example: Proteolytic purpose.
- Enzymes have the importance in the paper manufacturing. Examples: Endoxylanases for bleaching of Wood pulp.
- They can be used in the manufacturing of organic compounds. Example: Bacterial enzymes for the manufacturing of acetone, butanol, lactic acid etc.

(3)Enzymes used in food industry:-

Enzymes can be used in the meat packing industry. Example: Papain which is proteolytic in action, therefore hydrolyses peptide bonds thus for tenderizing meat and beef.

Enzymes have their role in Manufacturing of cheese. Example: Rennin (chymosin) found in stomach, converts milk protein casein to curd like calcium paracaseinate.

(4)Beverage industry:-

Papain is used to stabilize chill bear. Yeast enzymes are also used in beverage industry.

(5)Ice Cream:-

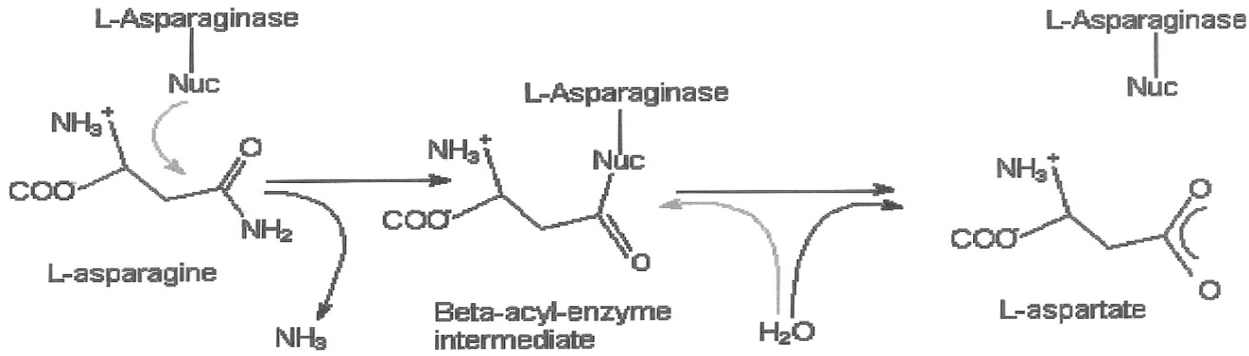
Lactose is used to prevent the formation of lactose crystals in ice-cream preparations.

(6)Research:-

Several enzymes are used for detection of biochemical reactions.

2. L-asparaginase: [4-16]

L-asparaginase (L-asparagine amino hydrolase EC 9.5.1.1) is a potent antitumor enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonium ion.



The chemical reaction of l-asparagine to l-aspartic acid and ammonia by L-asparaginase.

L-Asparagine + H_2O Asparaginase> L-Aspartate + NH_3

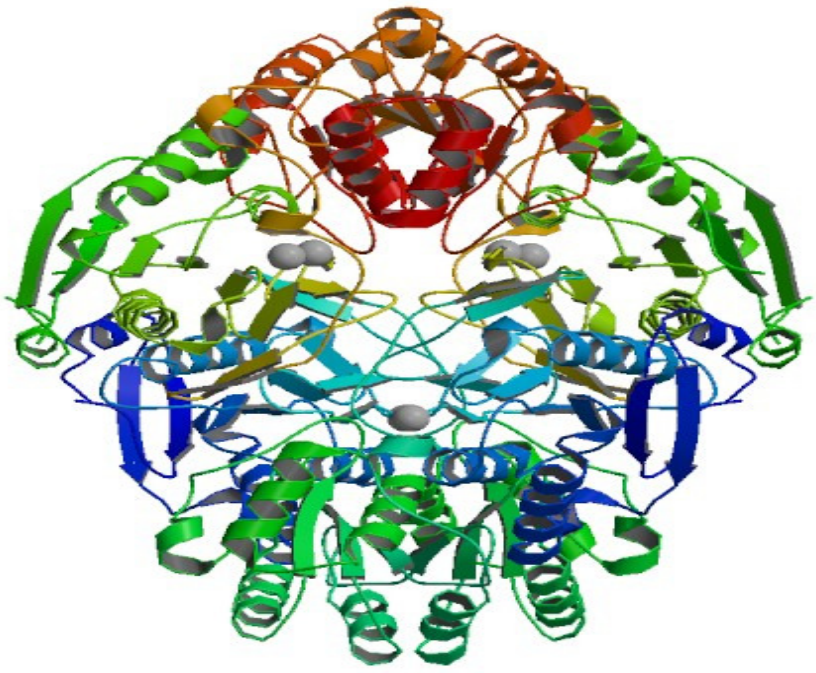
This enzyme has been widely exploited in the treatment of certain kinds of cancer especially acute lymphoblastic Leukemia since the time it was obtained from *Escherichia coli* and its antineoplastic activity demonstrated in guinea pig serum .[4-6]

Asparaginases are widely distributed in nature from bacteria to mammals and play a central role in the amino acid metabolism and utilization. In human body, L-aspartate plays an important role as a precursor of ornithine in the urea cycle and in transamination reactions forming oxalo acetate in the gluconeogenic pathway leading to glucose formation. [7]

L-asparaginase is a unique cancer chemotherapeutic agent [8]. It has been demonstrated that this enzyme is responsible for anti lymphoma activity in guinea pig serum. [9]

L-asparaginase is the first enzyme with anti leukemic activity to be intensively studied in human Beings [10]. Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine [11]. So, cancer cells are unable to produce L-asparagine and depend on external L-asparagine from the circulating blood plasma [12]. Since 1922, L-asparaginase has been considered as a therapeutic agent against malignant tumors [13-14]. A wide range of microorganisms such as filamentous fungi, yeasts, and bacteria have proved to be beneficial sources of this enzyme. [15-16]

STRUCTURE:



L-asparaginase structure

Figure: 1

SOURCE: [18]

L-Asparaginase is produced by a large number of microorganisms that include *E. coli* (Derst et al., 1994; Mercado & Arenas, 1999), *Erwinia cartovora*

(Maladkar et al., 1993; Aghaiypour et al., 2001*), Enterobacter aerogenes* (Mukherjee et al., 2000), *Corynebacterium glutamicum* (Juan et al., 1990), *Candida utilis* (Kil et al.1995), *Staphylococcus aureus* (Muley et al., 1998), *Thermus thermophilus* (Prista & Kyridio, 2001), and *Pisum sativum* (Siechiechowcicz & Ireland, 1989). [18]

MICRO-ORGANISM:

The Bacterial strain of *Pseudomonas aeruginosa* was collected from MTCC.

The stock culture was maintained on tryptic soy agar slants that contained tryptic casein Bios D (1.5%), Soy peptone (0.5%), NaCl (0.5%), and agar Bios U (1.5%). The culture was incubated at 37oC for 18 h and stored at 4oC.

MEDIA: [19]

Soybean Casein Digest Medium (Tryptone Soya Broth) :

Soybean Casein Digest Medium is a general purpose medium used for cultivation of a wide variety of microorganisms and Recommended for sterility testing of moulds and lower bacteria.

Composition:

Ingredients Gms / Litre

Pancreatic digest of casein 17.000 Gms

Papaic digest of soyabean meal 3.000 Gms

Sodium chloride 5.000 Gms

Dextrose 2.500 Gms

Dibasic potassium phosphate 2.500 Gms

Final pH (at 25°C) 7.3±0.2 Gms

FERMENTATION: [20]

Types of fermentation techniques

Solid-State Fermentation (SSF)

SSF utilizes solid substrates, like bran, bagasse, and paper pulp. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. In this fermentation technique, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. Hence, this technique supports controlled release of nutrients. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high aw (water activity), such as bacteria.(Babu and Satyanarayana, 1996).

Submerged Fermentation (SmF)/Liquid Fermentation (LF)

SmF utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture content. An additional advantage of this technique is that purification of products is easier. SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form. [20]

PURIFICATION:

Purification of L-asparaginase: [43-44]

The purification was carried out at 4ºC on the crude extract, according to the modified method of Distasio *et al*. (1976).

Ammonium sulfate fractionation

Finely powdered ammonium sulfate was added to a 80% saturation. The mixture was left for 12h at 4ºC, followed by centrifugation at 8,000 rpm for 20 min at 4ºC.

The precipitate was dissolved in a 0.01 M phosphate buffer pH 8.5 and dialyzed overnight against the same buffer at 4ºC.

The enzyme was assayed by the direct nesslerization method according to the method of Sinha *et al*., (1991). One L-asparaginase unit (IU) is defined as the enzyme amount, which liberates 1 µmol of the ammonia/min under optimal assay conditions. The protein concentration was determined by the modified Lowry's method (Kim *et al*., 2002).

Disc-PAGE: [18]

A slab gel electrophoresis was carried out using a 15% polyacrylamide gel (pH 7.8) with a 5% top gel (pH 6.2). After electrophoresis in a Tris-glycine buffer (pH 8.3) at 200 V for 7 h at 70oC, the proteins in the gel were stained with Coomassive brilliant blue R-250 and destained (El-Gamal *et al*., 2001). [18]

3. CURRENT STATUS ON THE PRODUCTION OF L-ASPARAGINASE:

The present research work focus on mutation, immobilization of the whole-cell *Pseudomonas aeruginosa* and optimize the fermentation medium (Soybean Casein Digest Medium) to produce cell free enzyme with high quality and quantity and low cost.

4. CANCER: [21]

Cancer is a potentially fatal disease caused mainly by environmental factors that mutate genes encoding critical cell-regulatory proteins (Malcolm R Alison, 2001). The resultant aberrant cell behaviour leads to expansive masses of abnormal cells that destroy surrounding normal tissue and can spread to vital organs resulting in disseminated disease; commonly a harbinger of imminent patient death, the cancer-causing agents (carcinogens) can be present in food and water, in the air, and in chemicals and sunlight that people are exposed to. [21]

HISTORY OF CANCER: [20]

Human beings and other animals have had cancer throughout recorded history. Cancer was discovered in Egypt and dates back to about 3000 BC. Although the term cancer was not used. The origin of the word cancer is credited to the Greek physician Hippocrates (460-370) He used the terms carcinos and carcinoma (which mean crab) to describe non-ulcer forming and ulcer forming tumors. Later the Roman physician Celsius (28-50AD) translated the term to cancer. [20]

CAUSES OF CANCER: [21]

During the 1970s, scientists discovered two particularly important families of genes related to cancer, oncogenes and tumor suppressor genes.

Oncogenes: These genes cause cells to grow out of control and become cancer cells. They are formed by changes or mutations of certain normal genes of the cell called *proto-oncogenes*. Proto-oncogenes are the genes that normally control how often a cell divides and the degree to which it *differentiates* (or specializes in a specific function in the body).

Tumor suppressor genes: These are normal genes that slow down cell division, repair DNA errors, and tell cells when to die (a process known as *apoptosis* or *programmed cell death*). When tumor suppressor genes don't work properly, cells can grow out of control, which can lead to cancer. [21]

CAUSES:

Different types of cancer have different causes and are likely to depend on many factors. Some cancers are more common than others, and chances for survival vary among different types. Most cancers do not have known causes from a chemical, environmental, genetic, immunologic, or viral origin. Cancers also can arise spontaneously from causes that are thus far unexplained.

The causes of cancer are very complex, involving both the cell and factors in the environment. Much progress has been made in identifying possible causes of cancer, including:

1-Chemicals and other substances: Being exposed to substances such as certain chemicals, metals, or pesticides can increase the risk of cancer. Any chemical that is known to cause cancer is called a carcinogen.

Example:

Asbestos, nickel, cadmium, uranium, radon, vinyl chloride, benzidene, and benzene

2-Tobacco: The most common carcinogens in our society are those present in cigarette smoke. Tobacco smoke is known to contain at least 60 carcinogens and 6 developmental

toxicants. In addition to being responsible for 80 to 90 percent of lung cancers, cigarette smoking is also associated with cancers of the mouth, pharynx, larynx, esophagus, pancreas, kidney, and bladder. Avoiding tobacco products is one way to decrease a person's risk of cancer.

3-Ionizing radiation: Certain types of radiation, such as x-rays, rays from radioactive substances, and ultraviolet rays from exposure to the sun, can produce damage to the DNA of cells, which might lead to cancer.

4- Heredity: Certain types of cancer occur more frequently in some families than in others, indicating some inherited predisposition to the development of cancer. Even in these cases, however, environment plays a part in the development of cancer. [22]

TYPES OF CANCER: [22]

Tumors are found in all kinds of tissue, and can be benign or malignant.

Benign tumors are not cancer: They usually can be removed and, in most cases, they do not come back. Most important, cells from benign tumors do not spread to other parts of the body. Cells from benign tumors stay together and often they are surrounded by a containing membrane. Benign tumors are not usually a threat to life**.**

Examples of Benign Tumors

Papilloma A projecting mass on the skin (for example, a wart)

Adenoma A tumor that grows in and around the glands

Lipoma A tumor in fatty tissue

Malignant tumors are cancer: Cancer cells can invade and damage tissues and organs near the tumor. Cancer cells also can break away from a malignant tumor and enter the lymphatic system or the bloodstream, which is how cancer can spread to other parts of the body. The characteristic feature of cancer is the cell's ability to grow rapidly, uncontrollably, and independently from the tissue where it started. The spread of cancer to other sites or organs in the body through the blood stream or lymphatic system is called metastasis.

Malignant tumors generally can be classified in two categories.

Carcinomas: These cancers originate in the epithelium. The epithelium is the lining cells of an organ. Carcinomas are the most common type of cancer. Common sites of carcinomas are the skin, mouth, lung, breast, stomach, colon and uterus.

Sarcomas: Sarcomas are cancers of connective and supportive tissue (soft tissues) of all kinds. Sarcomas can be found anywhere in the body, and they often form secondary growths in the lungs. [22]

Common Types of Cancer: [23]

Skin cancer is the most commonly diagnosed cancer among men and women. Over one million cases are diagnosed each year, with more young people having skin cancer than ever before. The most common types of cancer in the United States based on frequency of diagnosis are:

Bladder cancer

Breast cancer

Colon cancer

Endometrial cancer

Kidney cancer (renal cell)

Leukemia

Lung cancer

Melanoma

Non-Hodgkin lymphoma

Pancreatic cancer

Prostate cancer

Thyroid cancer

Types of Cancer Classified by Body System: [23]

Cancer has the potential to affect every organ in the body. The cells within malignant tumors have the ability to invade neighboring tissues and organs, thus spreading the disease. It is also possible for cancerous cells to break free from the tumor and enter the bloodstream, in turn spreading the disease to other organs. This process of spreading is called metastasis.

When cancer has metastasized and has affected other areas of the body, the disease is still referred to the organ of origination. For instance, if cervical cancer spreads to the lungs, it is still called cervical cancer, not lung cancer.

Blood Cancer: The cells in the bone marrow that give rise to red blood cells, white blood cells, and platelets can sometimes become cancerous. These cancers are leukemia or lymphoma.

Bone Cancer: Bone cancer is a relatively rare type of cancer that can affect both children and adults, but primarily affects children and teens. There are several types of bone cancer.

Brain Cancer: Brain tumors can be malignant (cancerous) or benign (non-cancerous). They affect both children and adults. Malignant brain tumors don't often spread beyond the brain. However, other types of cancer have the ability to spread to the brain.

Breast Cancer: Breast cancer is a type of cancer that affects women. More than 200,000 women are diagnosed with breast cancer in the United States each year.

Digestive/Gastrointestinal Cancers This is a broad category of cancer that affects everything from the esophagus to the anus. Each type is specific and has its own symptoms, causes, and treatments.

Endocrine Cancers: The endocrine system is an instrumental part of the body that is responsible for glandular and hormonal activity. Thyroid cancer is the most common of the endocrine cancer types and generally.

Eye Cancer: Like other organs in the human body, the eyes are vulnerable to cancer as well. Eye cancer can affect both children and adults.
Genitourinary Cancers: These types of cancer affect the male genitalia and urinary tract.

Gynecologic Cancers: These groups of cancer types affect the organs of the female reproductive system. Specialized oncologists called gynecologic oncologists are recommended for treating gynecologic cancer.

Head and Neck Cancer: Most head and neck cancers affect moist mucosal surfaces of the head and neck, like the mouth, throat, and nose. Causes of head and neck cancer vary, but cigarette smoking plays a role. Current research suggests a strong HPV_link in the development of some head and neck cancer.
Respiratory Cancers: Cigarette smoking is the primary cause for cancer affecting the respiratory system. Exposure to asbestos is also a factor.

Skin Cancers:

Non-melanoma skin cancer is the most common type of cancer among men and women. Exposure to the UV rays of the sun is the primary cause for non-melanoma skin cancer and also melanoma.

TREATMENTS OF CANCER: [24]

Cancer treatment is usually a combination of a number of different modalities. If the tumour is amenable to surgery, then surgery is the single most effective tool in the anticancer armamentarium. Targeted radiotherapy is another option, as are combinations of anticancer drugs. Most conventional anticancer drugs have been designed with deoxyribonucleic acid (DNA) synthesis as their Target. Therein lies the problem, in that tumour cells are not the only proliferating cells in the body such as bone marrow cells that generate red blood cells and cells to fight infection, and epidermal cells including those that generate hair are all highly proliferativeThus, patients with cancer receiving chemotherapy commonly suffer unwanted (hair loss) and sometimes potentially life-threatening (anaemia and proneness to infections) side effects that limit treatment. The new generations of drugs have targets removed from the direct synthesis of DNA; they affect the signals that promote or regulate the cell cycle, growth factors and their receptors, signal transduction pathways and pathways affecting DNA repair and apoptosis [23].

Anticancer drugs (1980) [25]

Table: 1

Alkylating agents	Nitrogen mustards: cyclophosphamide, melphalan, Chloroambucil Ethylsulfonates : busulfan Triazenes : dacarbazine Antibiotics : mitomycin-C
Antimetabolites	Folic acid analogs : methotrexate Pyrimidine analogs : 5-fluorouracil, cytarabine, 5-azacytidine Purine analogs : 6-mercaptopurine, 6-thioguanine Substituted ureas : hydroxyurea
Natural products	Antimitotics Vinca alkaloids: vincristine, vinblastine, Podophyllotoxins : etoposide, tenoposide Antibiotics : doxorubicin, daunorubicin, bleomycin, Actinomycin-D, mithramycin
Enzymes	L-Asparaginase
Hormones and antagonists	Adrenocorticosteroids: prednisone Progestins: hydroxyprogesterone caproate, medroxyprogesterone Acetate, megestrol acetate

	Estrogens: diethylstilboestrol, ethyniloestradiol
Miscellaneous agents:	Cisplatin, procarbazine, mitotan 1-asparaginase

Anticancer drugs (1999) [25]

Table: 2

Alkylating agents	Nitrogen mustards estracyte Oxazaphosphorines ifosfamide Hexitols mitobronitol Nitrosoureas photemustine Triazenes temozolomide, altretamine Aziridines mitomycin-C
Platinum complexes	Carboplatin, oxalyplatin
Topoisomerase inhibitors	Camptothecin topotecan, irinotecan Podophyllins etoposide, tenoposide
Antimetabolites	Folate analogs trimetrexate Purine analogs fludarabine Adenosine analogs cladribine, pentostatine Pyrimidine analogs floxouridine, gemcitabine, capecitabine
Antimitotics(microtubule agents)	Vincas vinorelbine Taxanes paclitaxel, docetaxel
Antibiotics	Glycopeptids pepleomycin Anthracyclins epirubicin, idarubicin, esorubicin Anthracenedions mitoxantron. Losoxantron
Hormonal agents	SnRH analogs octreotide, lanreotide GnRH analogs leuprolide, buserelin, goserelin, tryptorelin Aromatase inhibitors aminogluthetimide, fadrozole, vorozole, anastrozole, Letrozole, examestane, formestane Antioestrogens toremifene, raloxifene Antiandrogens flutamide, casodex Others fluoxymesterone, diethylstilboestrol

New approaches in cancer therapy (2000) [25]

Table: 3

Class	Mechanism	Agent	Investigational phase
Chemoprotective drugs	Transformation of precancerous to normal cell	All-trans-retinoic acid, etretinate, fenretinide, sulindac, raloxifene	II-III
Differentiating agents	Induction of cell differentiation	Phosphocholines, alkylglycerols, HMBA, DMSO, MAP	Preclinical + I
Revertants	Drugs bypassing MDR type resistance	Verapamil, dipins, sporins (e.g. staurosporin) various cytostatics	I-II
Natural products	various or not yet clarified	Plant extracts: (e.g. genistein, quercetin, chelidonin, marine products: Dolastaatin, bryostatin, halichondrin)	Preclinical + I-II
Supportive drugs	Cytoprotective agents – Hemopoiesis	IL-3, erythropoietin, G-CSF, thrombopoietin	II-III
	– Other organs	Mesna, amifostine, dexrazoxane	II-III
	Antibiotics	III generation cephalosporins, macrolides, antivirals (acyclovir, gancyclovir), Antimycotics (fluconazol)	III
	Analgesics	Morphine derivatives, fentanyl	
	Antiemetics	Ondansetron, tropisetron, granisetron, dolasetron	III
	Bisphosphonates	Chlodronate, etidronate, pamidronate, allendronate	III
Bioreductive agents	Tumour oxigenation	Tirapazamine	I-II

	enhancement		
Antitelomerases	Blocking telomerase	Telomerase inhibitors	Investigational
Angiogenesis	inhibitors Induction of vascular discontinuity Blocking endothelial locomotion Inhibiting endothelial proliferation Blocking endothelial sprouting	Metalloproteinase inhibitors, angostatic steroids Fumagillin + analogs, platelet factor 4 protamine Inhibitors of VEGF and FGF Marinastat, batimastat Thalidomid Angiostatin Endostatin Proline analogs, cyclosporine	Preclinical + I Preclinical + I-II I-II II-III II I Investigational II-III

REVIEW OF LITERATURE:

ENZYME:

- **Mirjafari et al** [4] **(2007)** Biological sequestration of carbon dioxide (CO_2) in geological formations is one of the proposed methods to reduce the carbon dioxide released into the atmosphere. In this method, an enzyme is used to enhance the hydration and subsequent precipitation of CO_2. In the present work, the effect of bovine carbonic anhydrase on the hydration of CO_2, and its precipitation in the form of calcium carbonate, was studied. The enzyme enhanced the hydration reaction. The rate of hydration reaction increased with both the enzyme concentration and temperature. The precipitation of calcium carbonate was promoted in the presence of the enzyme. The concentration of the enzyme did not affect the precipitation; however, temperature impacted the precipitation of calcium carbonate. At higher temperatures, less calcium carbonate was formed. Also, in the presence of the enzyme, calcium carbonate settled more quickly. The enzyme activity was not influenced by the pH of the reaction mixture. In contrast, the formation of calcium carbonate was affected by the pH of the solution. A kinetic analysis was performed for the bovine carbonic anhydrase. Based on the experimental results, the activation energy and catalytic rate constant are estimated as700.91 cal/mol and $0.65s^{-1}$, respectively.

L-ASPARAGINASE:

- **El-Bessoumy *et al*** [18] **(2004)** The L-asparaginase (E. C. 3. 5. 1. 1) Enzyme was purified to homogeneity from *Pseudomonas aeruginosa* 50071 cells that were grown on solid-state fermentation. Different purification steps (including ammonium sulfate fractionation followed by separation on Sephadex G-100 gel filtration and CM-Sephadex C50) were applied to the crude culture filtrate to obtain a pure enzyme preparation. The enzyme was purified 106-fold and showed a final specific activity of 1900 IU/mg with a 43% yield. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
- of the purified enzyme revealed it was one peptide chain with *Mw* of 160 kDa. A Line weaver-Burk analysis showed a *Km* value of 0.147 mM and *Vmax* of 35.7 IU. The

enzyme showed maximum activity at pH 9 when incubated at 37ºC for 30 min. The amino acid composition of the purified enzyme was also determined.

CANCER:

- **Malcolm R Alison** [21] (2001) Cancer is a potentially fatal disease caused mainly by environmental factors that mutate genes encoding critical cell-regulatory proteins. The resultant aberrant cell behaviour leads to expansive masses of abnormal cells that destroy surrounding normal tissue and can spread to vital organs resulting in disseminated disease, commonly a harbinger of imminent patient death.

- **American Society of Clinical Oncology** [22](2009)The study of cancer, called *oncology*, is the work of countless doctors and scientists around the world whose discoveries in anatomy, physiology, chemistry, epidemiology, and other related fields made oncology what it is today. Technological advances and the ever-increasing understanding of cancer make this field one of the most rapidly evolving areas of modern medicine.

- **www.atsdr.cdc.gov** [23] Cancer is not a single disease. It is a group of more than 200 different diseases. Cancer can be generally described as an uncontrolled growth and spread of abnormal cells in the body. Cells are basic units of life. All organisms are composed of one or more cells. Normally, cells divide to produce more cells only when the body needs them.
- Sometimes cells keep dividing and thus creating more cells even when they are not needed .When this happens, a mass of tissue forms. This mass of extra tissue is called a tumor. Tumors are found in all kinds of tissue, and can be benign or malignant.

SCREENING:

- **R.GULATI *et al*** [27] (1997) A pH and dye-based fast procedure for screening L-asparaginase producing micro-organisms is reported. The procedure is suitable for bacterial and fungal screening. The results are obtained within 24and48 hours for bacteria

and fungi, respectively .the results correlate with quantitative estimations in culture broths.

- **Dharmaraj *et al* [28] (2011)** L-asparaginase is an anti-neoplastic agent used in the chemotherapy of lymphoblastic leukaemia. The present work deals with production of extra-cellular Lasparaginase from marine actinomycetes, using submerged fermentation. Marine actinomycetes *Streptomyces* associated with marine sponge *Callyspongia diffusa* was isolated using specific ISP medium. Sponge-associated *Streptomyces* was characterized by conventional methods, and identified as *Streptomyces noursei* MTCC 10469. Production of Lasparaginase by submerged fermentation was carried out using medium Tryptone Glucose Yeast extract (TGY) broth. The enzyme was purified to near homogeneity by ammonium sulphate precipitation, dialysis, gel filtration on Sephadex G-100 column, CM Sephadex C-50 and SDS-PAGE. The enzyme was purified at 98.23 folds, and showed a final specific activity of 78.88 IU/mg, with 2.14% yield. SDS-PAGE of the purified enzyme revealed an apparent molecular weight of 102 kDa for it. The optimum pH, temperature and incubation time of L-asparaginase was found to be 8, 50ºC and 35 min, respectively. The study suggests that marine actinomycetes, particularly *Streptomyces*, may be used as a potential source of L-asparaginase.

- **Kamble *et al* [29]** (2012) Due to the hazards of chemotherapeutic drugs and its painful effects, L-asparaginase are emerging as safer source of anticancer enzymes. Genetic studies and enzyme purification studies has always been simpler with regards to bacteria hence bacteria are also preferred source for L-asparaginase production. Study so far reports some of the bacterial L-asparaginase is allergic and therefore there is a considerable need to find alternative bacterial L-asparaginase. We have employed a media containing L-asparagine as principle carbon source for screening of l-asparaginase producing bacteria. Twenty isolates were obtained after screening various soil samples. A qualitative assay was performed in order to select the efficient bacteria. Out of these isolates twelve efficient bacteria are further selected for physiological studies and carbon sources utilization. Optimization of pH and temperature is also studied. We have also performed a comparative assay of these isolates for Lasparaginase production. The

bacterial genera belong to *Paenibacillus species*, *B. subtilis, Aneurinibacillus species*, *Staphylococcus species*, *S. saprophyticus*, *Brevibacillus species* and *Micrococcus species*.

- **Kamble *et al* [30] (2012)** the habitat chosen for screening the bacteria were farm soil, saline soil and water. The activity was detected on a medium containing 1% peptone, 0.6% beef extract, 0.33% KH_3PO_4, 0.1% L-asparagine and phenol red. L-asparaginase activity was detected on the basis of formation of red colour around the colony. Likewise efficient L- asparaginase producing bacteria were screened. These were then studied for routine microbiological and biochemical characterization. The microorganisms we characterized belonged to the genera *E.coli, Serratia spp., Pseudomonas aeruginosa*, *Bacillus spp*., *Aeromonas species* and *Proteus spp.* L-asparaginase from halophilic bacteria is expected to be non-allergic and hence halophilic bacteria from saline soil can contribute to therapeutic value of this enzyme.

- **Verma *et al* [31] (2012)** Present scenario has focused that L- asparaginase has been found to be best anti leukemic agent. It has been characterized based on the assay principle hydrolyzing L-asparagine into L-aspartic acid and ammonia. *Withania somnifera* (L.) Dunal (Ashwagandha); Traditional ayurvedic medicinal plant, used in treatment of various diseases, found to have good source of enzyme L-asparaginase. In present study, different cytotypes of *W. somnifera* were compared for ploidy level and L-asparaginase activity. L-asparaginase was extracted from diploid and tetraploid *Withania somnifera* plants and tetraploid was found to have three times higher activity. Enzyme was purified using ammonium sulphate precipitation, Ion exchange and gel permeation chromatography. Purified L-asparaginase revealed molecular weight 75 kDa, Km value 1.5 mM and Vmax 526.31 μM/min. Purified enzyme has 61.22 % yield with 13.87 fold increase in specific activity

- **SUKUMARAN *et al* [32] (1979)** the production of L-asparaginase by two mutants of *Serratia marcescens* grown on 14 different media was studied. The enzyme content increased from trace levels to 2·4 international units per ml when the organisms were

grown in glycerol-peptone yeast extract medium. Glucose was the best carbon source under aerobic conditions. The enzyme content increased when L-asparagine was present in the growth medium.

- **Moharam *et al*** [33] **(2010)** L-asparaginase is one of the known drugs in the treatment of cancer, especially acute lymphoblastic leukemia. In recent years several bio-conjugation protocols have been developed to improve the pharmacokinetic and immunological properties of anti-leukemic enzyme, L-asparaginase. In this study, fifty two bacilli species were newly isolated in our laboratory and screened for their ability to produce extracellular and intracellular Lasparaginase enzyme. *Bacillus* sp R36 gave the highest intracellular enzyme production. Formation physiology of the enzyme revealed that optimum culture conditions were 9:1 of air::medium ratio, with 55 x 105 CFU/mL inoculums size. The optimum incubation period was found to be 24 hours under shaking growth conditions. The initial pH value 5.6 was favorable for the highest enzyme production. Addition of 1% lactose or 1% raffinose resulted in a doubled enzyme productivity (yielded 204% and 209%, respectively). The enzyme was efficiently immobilized by covalent binding with activated carbon. Immobilized L-asparaginase activity was 33.0 U/g carrier; with immobilization yield of 73.6%. Characterization of the enzyme was performed on native and immobilized forms. Optimum pH value was 7.0 for free and immobilized forms. Optimum reaction temperature was 50 °C for native enzyme, while it was 60 °C for the immobilized enzyme preparation. The immobilization process greatly enhanced the thermal stability of the enzyme. Native L -asparaginase enzyme exhibited thermal stability up to 50 °C, while immobilized form retained 100% of its activity up to 80 °C. Anti-tumor and antioxidant activities were investigated. The enzyme inhibited the growth of two human cell lines including hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116) with IC50 value of 112.19 μg/mL and 218.7 μg/mL, respectively.

MUTATION:

- **AMENA *et al*** [34]**(2012**) in the present study, the strain *Streptomyces gulbargensis* was subjected to mutagenesis by physical and chemical agents like UV rays, Ethyl methane sulphonate and Ethidium Bromide in order to isolate strains capable of producing higher yields of L-asparaginase than the prototype. A total of thirty two mutants were obtained, however among all the mutants, *S. Gulbargensis* mu24 was found to be the most potent. The maximum L-asparaginase activities recorded with *S. gulbargensis* and its mutant were 30 IU and 44.7 IU respectively at 120 h of fermentation using groundnut cake extract as medium. Hence, L-asparaginase production was enhanced by 1.49 fold by mutation. Also, attempts were made to enhance the L-asparaginase production of the prototype and the mutant strains by whole-cell immobilization in gelatin. The medium with an initial pH 8.5 was inoculated with free and immobilized cells separately and subjected to fermentation at 40^{o}C at 200 rev/min. In an immobilized cell system, the enzyme production was enhanced by 1.10 fold compared to the conventional free-cell fermentation. The immobilized cells retained their ability to produce L-asparaginase over three cycles and the activity remained between 49.4 IU-18.3 IU (mutant strain) and 33.15 IU-12.9 IU (prototype) throughout the three cycles, with the highest activity obtained during the first cycle. Hence, the gelatin-immobilized cells of *S.gulbargensis* mu24 can be explored as an effective biocatalyst which can be repeatedly used for maximum production of L-asparaginase.

- **Sreenivasulu *et al*[35] (2009)** The purpose of the present investigation is to enhance production of chemotherapeutically important anti cancer enzyme L-asparaginase by subjecting the indigenous L-asparaginase producing strain *Aspergillus sp*. VS-26 to improvement by natural selection and random mutagenesis [UV and ethyl methyl sulphonate, (EMS)] .The isolation of mutants and the enzyme activity of selected mutants were described. The best natural selectant VNS-8 showed 2.2% higher L-asparaginase activity than the wild strain (VS-26). The L-asparaginase yield of the best UV mutant VUV-5 was 31.7% higher than the parent strain (VNS-8) and 34.6% higher than the wild strain (VS-26). Also, the L-asparaginase yield of the best EMS mutant VEM-9 was 38.8 % higher than the parent strain (VUV-5) and 87 % higher than the wild strain (VS-26). The results were indicated that UV and EMS were effective mutagenic

agents for strain improvement of *Aspergillus SP.* VS-26 for enhanced antitumor L-asparaginase productivity.

IMMOBILIZATION:

- **Deepali BISHT *et al*** [36] Suitability of three matrices *viz.* agarose, sodium alginate and polyacrylamide for immobilization of mutant cells of *Pseudomonas aeruginosa* MTCC 10,055 was investigated. Of these, agarose was proved to be the best exhibiting maximum enzyme production (4363.4 U/mL) followed by polyacrylamide gel (2172.3 U/mL) whereas, alginate beads were the poorest. The one-variable-at-a-time approach suggested agarose, 2.0%; immobilized bead, 4.0 g blocks/50 ml and initial cell loading of 0.8 g in the matrix as optimum conditions for maximum lipase production (5982.3 U/mL) after 24 h of incubation. However, RSM studies determined the optimum values of these variables as 1.96%, 4.06 g blocks/50 mL and 0.81 g cells in matrix for maximum lipase production (6354.23 U/mL) within 22.54 h of incubation. The agarose blocks were reusable for seven cycles without any significant loss in lipase yield. Bench-scale bioreactor level optimization resulted in further enhancement in lipase yield (6815.3 U/mL) at 0.6 vvm aeration and 100 rpm agitation within only 20 h of incubation. Presumably, this is the first attempt on lipase production by immobilized cells of *P.aeruginosa* at bioreactor level. The agarose immobilized mutant cells claimed its potential candidature for alkaline lipase production at industrial level.

- **Kattimani *et al*[37] (2009)** in the present study, *Streptomyces gulbargensis* and its mutant form, *S.gulbargensis* mu24, immobilized on polyurethane foam were investigated for the production of L-asparaginase using groundnut cake extract as medium. The medium with an initial pH of 8.5 was inoculated with free and immobilized cells separately and then subjected to fermentation by incubation at 40oC and shaking at 200 rev/min. In the immobilized cell system, enzyme production was enhanced by approximately 30%

compared to the conventional free cell fermentation. The immobilized cells were subjected to repeated batch fermentation processes to determine their reusability. These cells retained their ability to produce L-asparaginase over seven cycles and the activities remained between 16.2-41.3 IU/ml and 39-60 IU/ml for *S.gulbargensis* and *S.gulbargensis* mu24, respectively. The maximum enzyme titer was obtained during the third batch by both strains. However, the mutant strain was more potent for L-asparaginase production than the prototype. Therefore, the polyurethane foam immobilized cells of *S.gulbargensis* mu24 can be proposed as an effective biocatalyst, which can be repeatedly used for maximum production of L-asparaginase.

PRODUCTION:

- **SUNDARAMOORTHI *et al*** [38] (2012) Enzymes are proteins that catalyze (*i.e.*, increase the rates of) chemical reactions. The manufacture of an enzyme for use as a drug is an important facet of today's pharmaceutical industry. Microbial L-Asparaginase (L-Asparaginase amido hydrolase) has been widely used as a therapeutic agent in the treatment of certain human cancers, mainly in acute lymphoblastic leukemia. Thermophilic fungi can be grown in minimal media with metabolic rates and growth yields comparable to those of mesophilic fungi. L-asparaginase perform essential role in the treatment of acute lymphoblastic leukemia, lymphosarcoma and in many other clinical experiments relating to tumour therapy in combination with chemotherapy. Fungal isolates were isolated from soil samples collected from different regions of the Arabian Sea, using potato dextrose agar (PDA) medium by serial dilution method. The inoculated agar plates were incubated at 37˚C for 4 to 7 days. Twelve isolates were selected and the isolated strains were screened by plate assay method using Czapek Dox medium and potential strains were used for the production of L-Asparaginase. It was found out that among the twelve isolates five showed significant production. One IU of L-Asparaginase is the amount of enzyme which liberates 1µmol of ammonia per minute per ml [µmole/ml/min]. From this work we conclude that more than 80% of the fungal strains from marine soil sample had the ability to produce the enzyme L-Asparaginase. The highest immobilized activity and highest immobilization yield were obtained with Silica gel carrier.

- **Rashmi *et al* [39] (2012)** Pseudomonas aeruginosa BGNAS-5 a locally isolated bacteria was selected for production of L-glutamianse in minimal fermentation broth. The optimization of fermentation process reveals that the maximum enzyme activity 46 IU at pH 7, 48 IU at temperature 360C, 36 IU at 72 hours incubation, 64 IU maltose as carbon source and 62 IU glutamin as nitrogen source. With this optimized fermentation broth Pseudomonas aeruginosa BGNAS-5 explored as promising organism for the production of an enzyme Lglutaminase used as anticancer agent.

- **Saleem Basha *et al* [40] (2009)** the objective of this investigation was to isolate marine actinomycetes, screen them for Lasparaginase activity and characterize the enzyme. Marine actinomycetes were isolated from sediment samples obtained from Tamilnadu and Kerala in India. The isolates were identified as actinomycetes by microscopical and biochemical tests. Production of L-asparaginase was carried out in three different media, namely, solid-state media, Tryptone Glucose Yeast extract (TGY) broth and Tryptone Fructose Yeast extract (TFY) broth.. The enzyme was purified to near homogeneity by ammonium sulphate precipitation, dialysis, gel filtration on Sephadex G-100 column and SDS-PAGE. Among 10 marine isolates subjected to preliminary screening, only isolates S3, S4 and K8 showed potential for L-asparaginase activity. All three marine soil isolates synthesized asparaginase with yield ranging from 24.6 to 49.2 IU/ml. Soil isolate S3 showed the highest productivity of 49.2 IU/ml with a protein content of 65 µg/ml and optimum activity at pH 7.5 and 50 ºC. The apparent Km value for the substrate was 25 µM. Mg^{2+} ion slightly stimulated activity while Cu^{2+}, Zn^{2+} and EDTA were inhibitory. The study revealed that marine actinomycetes may be a potential source of high yield.

- **Manikandan *et al* [41] (2010)** Evaluation of fermentation process parameter interactions for the production of l-asparaginase by *Pseudomonas aeruginosa*. Box-Behnken design of experimentation was adopted to optimize nutritional sources, physiological (incubation period) and microbial (inoculum level).The experimental results and software predicted enzyme production values were comparable. Incubation period, inoculum level and

nutritional source (soybean) were major influential parameters at their individual level. Interaction data of the selected fermentation parameters could be classified as least and most significant at individual and interactive levels. All selected factors showed impact on L-asparaginase enzyme production by this isolated microbial strain either at the individual or interactive level. Incubation temperature, inoculums concentration, and nutritional source (soybean) had impact at individual level. Significant improvement in enzyme production by this microbial isolate was noted under optimized environment.

- **HEINEMANN *et al*[42] (1969)** Production of a tumor-inhibitory asparaginase by submerged fermentation with *Serratia marcescens* ATCC 60 was studied to ascertain optimal nutritional conditions for large-scale production leading to enzyme purification studies. Five strains of S. marcescens were screened in shake-flask studies and were found to produce 0.8 to 3.7 IU/ml 48 hr after inoculation. The requirements for asparaginase production with S. marcescens ATCC 60, the high producing strain, included the following: 4% autolyzed yeast extract medium (initial pH 5.0), an incubation temperature of 26 ^{0}C, and limited aeration for a zero level of dissolved oxygen during the fermentation. Addition of various carbohydrates to the fermentation medium did not enhance yields. The peak cell population in the fermentation medium and the maximal asparaginase yields occurred simultaneously. Highest enzyme yields were found when the pH of the fermentation cycle rose to approximately 8.5. Yields of 4 IU of asparaginase/ml of cell suspension have been obtained consistently in 40 to 42 hr from 10-liter volumes (500 ml/4-liter bottle) produced on a reciprocating shaker. Scale-up to a 60-liter fermentor yielded 3.1 IU/ml in 35 hr.

PURIFICATION:

- **Suchita *et al* [43] (2010)** L-asparaginase was extracted from *Erwinia carotovora* and purified by ammonium sulfate fractionation (60–70%), Sephadex G-100, CM cellulose, and DEAE Sephadex chromatography. The apparent Mw of enzyme under none denaturing and denaturing conditions was 150 kDa and 37 ± 0.5 kDa, respectively. L-asparaginase activity was studied in presence of thiols, namely, L-cystine (Cys), L-methionine (Met), N-acetyl cysteine (NAC), and reduced glutathione (GSH). Kinetic parameters in presence of thiols (10–400 μM) showed an increase in Vmax values (2000,

2223, 2380, 2500, and control 1666.7 μmoles mg−1min−1) and a decrease in K*m* values (0.086, 0.076, 0.062, 0.055 and control 0.098mM) indicating nonessential mode of activation. KA values displayed propensity to bind thiols. A decrease in Vmax/K*m* ratio in concentration plots showed inverse relationship between free thiol groups (NAC and GSH) and bound thiol group (Cys and Met). Enzyme activity was enhanced in presence of thiol protecting reagents like dithiothreitol (DTT), 2-mercaptoethanol (2-ME), and GSH, but inhibited by p-chloromercurybenzoate (PCMB) and iodoacetamide (IA).

- **Balasubramanian *et al* [44] (2012):** L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an extra cellular enzyme that has received considerable attention since it is used as an anticancer agent. In the present study, the fungal isolates from marine soils were screened for the L-asparaginase production by using modified Czapek Dox agar containing L-asparagine and phenol red as indicator. This production of L-asparaginase was produced through submerged fermentation by using isolates *Aspergillus terreus*. This production of L-asparaginase was achieved through optimization of fermentation parameters and it showed maximum of enzyme activity. After the production of L-asparaginase were used for purification by chromatography techniques. The purified L-asparaginase was used for the characterization such as HPLC assay of L-asparaginase activity and Molecular weight determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme revealed it was one peptide chain of 94 kDa. The amino acid composition of the purified enzyme was also determined.

ASSAY OF L-ASPARAGINASE:

- **GAFFAR *et al* [46]** (1977) Asparaginase was found in the soluble fraction of cells of Azotobacter vinelandii, and its activity remained the same during growth of the organism in a nitrogen-free medium. The specific activity and the yield of A. vinelandii Increased twofold in the presence of ammonium sulfate. Within limits, the Temperature (30 to 37°C) and pH (6.5 to 8.0) of the medium showed little effect on the levels of enzyme activity. The enzyme was purified to near homogeneity by Standard methods of enzyme purification, including affinity chromatography, and had optimum activity at pH 8.6 and 48°C. The approximate molecular weight was 84,000. The apparent K, value for the substrate was 1.1 x 10-4 M. Metal ions or sulfhydryl reagents were not required for

enzyme activity. Cu^{2+},Zn^{2+}, and Hg^{2+} showed concentration-dependent inhibition, whereas amino and keto acids had no effect on the enzyme activity. Asparaginase was stable when Incubated with rat serum and ascites fluid. The enzyme had no effect on the membrane of sheep erythrocytes and did not inhibit the incorporation of radioactive precursors into deoxyribonucleic acid, ribonucleic acid, and protein in Yoshida ascites sarcoma cells. Asparaginase activity was not detected in the tumor cells.

- **Thirunavukkarasu *et al*[47] (2011)** Symptomless endophytic fungi isolated from seven green algae, six brown algae and six red algae occurring along the southern coast of Tamilnadu, southern India were screened for the production of L-asparaginase enzyme. Of the 82 endophyte isolates, 64 were positive for the enzyme. A *Fusarium* sp. isolated from the thallus of *Sargassum wightii* and a sterile mycelial form isolated from the thallus of *Chaetomorpha* sp. showed maximum activity of the enzyme. The mycelia growth of *Fusarium* sp. was positively correlated with enzyme production. In a time course study, maximum enzyme activity was observed on the 5^{th} day of growth of this fungus. The optimum pH for enzyme activity was pH 6.2. High concentration of glucose in the medium as C source inhibited enzyme production by the fungus. Endophytes of tropical seaweeds appear to be a good source of this therapeutic enzyme.

- **Borah *et al*** [48] (*2012*) Asparaginase s are anti-cancer agent used in the lymphoblastic leukemia chemotherapy. Asparaginase is hydrolytic enzyme and is produced by a large number of microorganisms. The production of asparaginase enzyme was carried out by using L-Asparagine as a substrate with the help of *E. coli*, isolated from sewage. The enzyme was purified by salt precipitation, dialysis and ion exchange chromatography. The enzyme activity was found to be 0.039 U/ml/min and the molecular weight was determined as 153 KDa with the help of SDS-PAGE. The Optimum temperature and pH was recorded as $55^{O}C$ and 6 respectively. The study has shown the capability of the enzyme to withstand high temperature and hence can be considered as thermo stable enzyme.

- **Siddalingeshwara *et al* [49] (2011)** this paper describes production and characterization of L-asparaginase from *Aspergillus terreus* KLS2. This L-asparaginase was produced through solid state fermentation by using carob pod as a substrate. This production of L-asparaginase was achieved through optimization of fermentation parameters and it showed 6.05 IU of enzyme activity. After the production of L-asparaginase were used for purification by chromatography techniques. The purified L-asparaginase was used for the characterization. The general properties were used for characterization of L-asparaginase is effect of pH and temperature, stability of pH and temperature on L-asparaginase. Even the substrate specificity was also studied. The optimum pH 9.0 retains 100% of residual activity and 45^0C temperature on Lasparaginase retains 100% residual activity. Stability of pH 8.0 retains 100% activity and temperature 70 ^{0}C at 30 and 60 min. L-asparagine was used as a specific and natural substrate to the L-asparaginase and retain 100% relative activity.

SDS-PAGE:

- **Jayaramu *et al* [51]** (2010) L-Asparaginase is an important component in the treatment of acute lymphoblastic leukemia in children. The manufacture of L-asparaginase by *Emericella nidulans* were carried out by novel flask method for screening. Using modified Czapek Dox both containing L-asparagine and phenol red as indicator. L-asparaginase productions were detected by change into pink colour in flask. Further confirmations of L-asparaginase synthesis were carried out by thin layer chromatograph. Production of L-asparaginase was carried out by using pH, temperature and inoculums sizes for optimization process. The pH 6, temperature 30 ^{0}C and 0.75 ml of inoculums were used.

- **Saravanan *et al* [52] (2011)** L-asparaginase is an important chemotherapeutic agent used for the treatment of a variety of lymphoproliferative disorders, lymphomas and acute lymphoblastic leukemia. In recent years, the use of L-asparaginase in the treatment of leukemia and other lymphoproliferative disorders has expanded immensely. In view of the potential applications of L-asparaginase and the need for development of economical methods for improved enzyme production with an overall aim of reducing the

cost of the industrial process, solid state fermentation (SSF) can serve as an excellent alternative for increasing enzyme yields. Hence present investigation was aimed at exploring the synthetic ability of Lasparaginase from *Pectobacterium carotovorum* (MTCC 1428) and *Bacillus circulans* (MTCC 490) by providing suitable synthetic mediums.

OTHER SUPPORTIVE LITERATURE:

- **Hegazy *et al* [53] (2012)** this work aimed to characterize recombinant strains, with a high production level of L-asparaginase, previously obtained by protoplast fusion between *Bacillus subtilis* and *Bacillus cereus*. The genetic relationship between fusants and their parents was determined using a simple PCR restriction fragment length polymorphism (RFLP) of 16S rDNAs -based method, and by analyzing the specifi c L-asparaginase genes and protein patterns. Our results indicate that all fusants have morphological characteristics and specific L-asparaginase genes similar to the *Bacillus cereus* strain whereas protein pattern and 16S rDNAs -RFLP analysis show mixed characteristics from both parents. Different genetic relationships were found between each fusant and its parental strain. These results confirm that protoplast fusion is an important technique in strain improvement. It was used to combine genes from different organisms for creating strains with desired properties.

- **Kavitha *et al* [54] (2012)** an enzyme-based drug, L-asparaginase, was produced by *Nocardia Levis* MK-VL 113 isolated from laterite soils of Guntur region. Cultural parameters affecting the production of L-asparaginase by the strain were optimized. Maximal yields of L-asparaginase were recorded from 3-day-old culture grown in modified asparagine-glycerol salts broth with initial pH 7.0 at temperature 30 ^{0}C. Glycerol (2%) and yeast extract (1.5%) served as good carbon and nitrogen sources for L-asparaginase production, respectively. Cell-disrupting agents like EDTA slightly enhanced the productivity of L-asparaginase. Ours is the first paper on the production of L-asparaginase by *N. Levis*.

- **Jha *et al* [55] (2012)** L-Asparaginase (E.C. 3.5.1.1.), also known as L-asparagine amidohydrolase is the enzyme with anti-tumor activity and is well accepted as a chemotherapeutic agent against the acute lymphoblastic leukemia and lymphosarcoma. This article has briefly touched nearly all the industrial and clinical aspects of L-Asparaginase and provides the recent update of the topic. The article includes a brief introduction to the topics, mechanism of action, a little information about the structure, sources of enzyme, purification, optimum conditions for the enzyme production, recombinant strains for higher productivity and formulation of the enzyme.

SCOPE OF WORK

Since the observation Ashraf A. El-Bessoumy et al that L-asparaginase from *Pseudomonas aeruginosa* has antitumor activity similar to that of guinea pig serum which discovered by Kidd (1953) few numbers of papers have been published on the production of L-asparaginase by *Pseudomonas aeruginosa.* Thus, a large amount of L-asparaginase to be supplied to cure so many patients around the world whose are suffering from different types of cancers such as leukemia and other malignant neoplasms in human. However the administration of L-asparaginase for a long time cause production of antibodies which causes anaphylactic shock or neutralization to the cancer patient, therefore, new L-asparaginase with different immunological effect to be discover to avoid the anaphylactic shock. To produce such enzyme requires some changes in parameters, strain and optimization of the medium.

OBJECTIVE OF WORK

There are some researches has been done on biosynthesis L-Asparaginase using *Pseudomonas aeruginosa* as a source. The fermentations were carried out by solid state fermentation technique but there are some researches had proved that submerged fermentation technique is better than solid state fermentation technique for bacteria and fungi because they require moisture for their growth.

The present research work focus to increase the production of L-asparaginase By modifying the fermentation techniques. The following steps were carried out to improve the productivity of L-asparaginase:

1. Irradiation
2. Immobilization of the whole-cell *Pseudomonas aeruginosa*
3. Optimize the fermentation medium (Soybean Casein Digest Medium) by adding phosphate buffer
4. Submerged fermentation technique will be use for the production
5. Confirmation of the product will be carried out by SDS-PAGE

PLAN OF WORK

1. Collection of the strain culture
2. Screening the Culture for *L-Asparaginase* by Rapid plate method.
3. Irradiation
4. Immobilization of Whole-Cell
5. Fermentation (Submerged Fermentation)
6. Assay of L-asparaginase from crude extract
7. Isolation
8. Purification
9. Characterization of L-asparaginase

   Effect of pH on enzyme activity

   Effect of temperature on enzyme activity
10. Conformation of enzyme production by SDS-PAGE

MATERIALS AND METHODS

LIST OF CHEMICALS USED:

Table: 4

Sl No	Chemical	Company
1	Soyabean meal broth	Himedia
2	Nutrient broth	Himedia
3	Nutrient agar	Himedia
4	L-asparagine	Himedia
5	Grams iodine	Otto kemi
6	Malachite green	Nice chemical
7	Methylene blue	Otto kemi
8	Safranin	Nice chemical
9	Nesseler's reagent	Reachem
10	Immersion oil	Qualigens
11	Ferrous sulphate	Nice chemicals
12	Zinc sulphate	Qualigens
13	Magnesium sulphaate	Micro fine chemicals
14	Cupper nitrate	Merck
15	Potassium chloride	Merck
16	Potassium dihydrogen phosphate	Himedia
17	Sodium chloride	Merck
18	Sodium hydroxide	Finar
19	Sodium alginate	Otto kemi
20	Magnesium chloride	Qualigens
21	D-glucose	Qualigens
22	D-fructose	Qualigens
23	Sucrose	
24	Agar-agar	SRL
25	Phenol red dye	Reachem
26	Sulphuric acid	Finar
27	Hydrochloric acid	Finar
28	Trichloro acetic acid	SRL
29	Absolute alcohol	Jiangsu huaxi international
30	Ammonium per sulphate	SRL
31	Bromo thymol blue	Merck
32	TE buffer	Medox
SI no	**Chemical**	**Company**
34	Phosphate buffer	

35	Ammonium sulphate	Qualigens
36	Acrylamide	SD fine
37	Bis acrylamide	Qualigens
38	Petroleum jelly	Himedia
39	Casein	Himedia

LIST OF INSTRUMENTS USED:

Table: 5

SI no	Instrument	Manufacturer	Brand
1	Electronic balance	Shimadzu AY 220 Max-220 g,min-10mg	
2	Electronic balance	Shimadzu AUX 220 Max-220 g,min-10mg	Unibloc
3	Hot air oven	Ashok united scientific company	
4	Colorimeter	Aimil Ltd	
5	Water bath	Industrial and laboratory tools corporation	
6	Centrifuge	Remi	R8C
7	Centrifuge	Remi	RM12C
8	Centrifuge	Remi	R24
9	Shaker	Scigenics	Orbitek

Sl no	Instrument	Manufacturer	Brand

10	Incubator	Industrial and laboratory tools corporation	S.L-SZ-08
11	Horizontal laminar airflow cabinate	Air tech	
12	Vertical SDS-PAGE electrophoresis	Medox	
13	Gel documentation system	Vilber lourmat	V03-7833
14	Autoclave	Medica instrument Mfg	Equitron
15	Microwave oven	Whirl pool	
16	Glass ware	Qualigens	
17	Motor stirrer	Kadavil electro mechanical industries	
18	Rocker	Scigenics	Orbitek
19	Microscope	Micron optik	Bino cxi
20	Vortex		Spinix
21	Refrigerator	Sub Zero lab instruments	

METHODOLOGY

1. COLLECTION OF THE STRAIN CULTURE:

The Bacterial strain of *Pseudomonas aeruginosa* was collected from MTCC Chandigarh.

2. SCREENING THE CULTURE FOR L-ASPARAGINASE BY RAPID PLATE METHOD [27]:

The strain was screened for L-asparaginase production using a method in which modified M9 medium (composition for 1 l: 6.0 g $Na_2HPO4{\cdot}2H_2O$; 3.0 g $KH2PO_4$; 0.5 g $NaCl_2$; 5.0 g L-asparagine; 1mole/litre of 2.0 ml $MgSO_4.7H_2O$; 0.1mole/litre of 1.0ml $CaCl_2.2H_2O$; 10 ml of 2.0% w/v glucose; and 20.0 g agar) incorporated with a pH indicator (phenol red) was used (Gulati *et al*., 1997). L-asparaginase activity was identified by formation of pink zone around colonies.

Media: Modified M9 Media

Composition of the Modified Media [28]:

Table: 6

Chemicals	Amount for 1000ml
Disodium hydrogen phosphate.$2H_2O$	6.0g
Potassium di hydrogen phosphate.	3.0g
Sodium chloride	0.5g
L-asparagine	5.0g
Magnesium sulphate(1mole/litre)	2.0ml
Calcium chloride(0.1mole/litre)	1.0ml
20% glucose stock solution	10.0ml
Agar	20.0g
Distilled water	1000ml

Media pH -7.0

Preparation of various concentration of dye [29]:

1- 2.5% stock solution of phenol red dye was prepared in ethanol and the pH was adjusted to 7.0 using 1mole/litre of sodium hydroxide.

2- The stock solution of the dye, ranging from 0.04ml to 0.3ml, was added to 1000ml of modified M9 medium, given final dye concentrations of 0.001% to 0.009% respectively.

Procedure [30-33]:

1-20ml of 0.005% of the above dye containing media was poured into a Petri plates.

2- Two control plates were also prepared by using modified M9 medium .Among them one was without dye and the other one was without asparagine (instead containing $NaNO_3$ as nitrogen source).

3- The strain was inoculated in Petri plates.

4- The plates were incubated for 18hrs at 37°C.

5- The zone diameter where measured after 18hrs of incubation

3. IRRADIATION OF THE STRAIN [34-35]:

The strain has been mutated by Physical method (UV irradiation), the mutation of the strain was done by exposing the strain to UV irradiation from different distances for different times as in the following column.

Table: 7

S,NO	DISTANCE FROM THE UV LAMP In 5cm			DISTANCE FROM THE UV LAMP In 10cm			DISTANCE FROMTHE UV LAMP In 15cm		
	TIME OF EXPOSURE			TIME OF EXPOSURE			TIME OF EXPOSURE		
1	10	20	40	10	20	40	10	20	40
2	10	20	40	10	20	40	10	20	40
3	10	20	40	10	20	40	10	20	40
4	10	20	40	10	20	40	10	20	40
5	10	20	40	10	20	40	10	20	40
6	10	20	40	10	20	40	10	20	40
7	10	20	40	10	20	40	10	20	40
8	10	20	40	10	20	40	10	20	40
9	10	20	40	10	20	40	10	20	40

4. IMMOBILIZATION OF WHOLE CELL [36-37]:

The culture strain has been immobilized by gel entrapment technique using sodium alginate as supporting material. The growing bacterial cells were harvested from culture broth by centrifugation at 12000×*g* for 10 min. The supernatant was removed and the pellet (0.2g) was washed twice with sterile distilled water and mixed with sterilized 20 mL of 4 %(w/v) sodium alginate prepared in distilled water. The slurry was extruded through a syringe into 0.2 M calcium chloride solution with constant stirring. The beads were allowed to cure in 0.2 M calcium chloride solution for 24 h at 4 °C followed by washing with sterile distilled water for 3 to 4 times and preserved in Tris-HCl buffer (pH-8.0) at 4 °C until use.

5. FERMENTATION (SUBMERGED FERMENTATION) [38-39]:

Submerged fermentation was carried out by modified minimal fermentation broth that contained an amount of Soybean Casein Digest Medium broth. Comprising of Pancreatic digest of casein 1.7gm, Papaic digest of soyabean meal 0.3gm, Sodium chloride 0.5gm, Dextrose 0.25gm, dibasic potassium phosphate 0. 25gm in 100ml of water and Final pH (at 25°C) 7.3±0.2 then contained in a 250 ml Erlenmeyer flask, was inoculated separately with the mutants and wild strain of *Pseudomonas aeruginosa* and incubated at 37 ºC in a shaker at 200 rev/min for 3-6 days. At the end of the fermentation period, the crude enzyme was prepared by centrifugation at 10000 rpm for 20 min. The cell-free supernatant was taken as the crude enzyme.

6. ASSAY OF L-ASPARAGINASE FROM CRUDE EXTRACTS [40-42]:

Assay of enzyme was carried out as per Imad et al, 0.5 ml of 0.04 M asparagine was taken in a test tube, to which 0.5 ml of 0.5 M buffer (acetate buffer pH 5.4), 0.5 ml of enzyme and 0.5 ml of distilled water was added to make up the volume up to 2.0 ml and incubate the reaction mixture for 30 min. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). 0.1 ml was taken from the above reaction mixture and added to 3.7 ml distilled water and to that 0.2 ml Nessler's reagent was added and incubated for 15 to 20 min. The OD was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA. The enzyme activity was expressed in International unit.

International Unit (IU)

One IU of L-asparaginase is the amount of enzyme which liberates 1 µmol of ammonia per minute per ml [µmole/ml/min].

$$\text{Units/mL enzyme} = \frac{(\mu\text{mole of } NH_3 \text{ liberated})\ (2.5) \times 100}{(0.1)\ (30)\ (1)}$$

2.5 = Initial volume of enzyme mixture (mL)

0.1 = Volume of enzyme mixture used in final reaction (mL)

30 = Incubation time (minutes)

1 = Volume of enzyme used (mL)

7. ISOLATION [18]:

The extracellular crude enzyme was prepared at the end of the fermentation period by the addition of 90 ml of a 0.01 M phosphate buffer pH 7 to the fermented medium, shaking for 15

min followed by centrifugation at 8,000 rpm for 20 min. The cell free supernatant was used as the crude enzyme preparation.

8. Purification [43-44]:

The purification was carried out at 4ºC on the crude extract, according to the modified method of Distasio *et al*. (1976).

Ammonium sulfate fractionation: Finely powdered ammonium sulfate was added to 80% saturation. The mixture was left for 12 hrs at 4ºC, followed by centrifugation at 8,000 rpm for 20 min at 4ºC.The precipitate was dissolved in a 0.01 M phosphate buffer pH 8.5 and dialyzed overnight against the same buffer at 4ºC.

9. ESTIMATION OF L-ASPARAGINASE BY LOWERY METHOD [45]:

This procedure is sensitive because it employs two colour forming reactions. It uses the Biuret reactions in which Cu2+ in presence of a base reacts with a peptide bond of protein under alkaline conditions resulting in reduction of cupric ions (Cu2+) to cuprous ions (Cu+), and Lowry's reaction in which the Folin Ciocalteau reagent which contains phosphomolybdic complex which is a mixture of sodium tungstate, sodium molybdate and phosphate, along with copper sulphate solution and the protein, a blue purple colour is produced which can be assessed by measuring the absorbance at 650 nm

Materials Required:

1) Standard Protein solution [1mg/ml].
2) Alkaline copper reagent.
3) Folin's Ciocalteau reagent.
4) Colorimeter
5) Pipettes.

PROCEDURE:

Arrange the reagent solutions prepared, on the table.

Label the test tubes with the volume taken and arrange them in a test tube rack.

Pipette out the standard protein solution from the standard flask into the test tubes labelled [0.2ml-1ml].

Pipette a known volume of the unknown solution to the tube labelled “unknown” arranged in the test tube rack.

To the test tube labelled ‘Blank’, add 1ml of distilled water using a micropipette.

Volume in each test tube is made up to 1 ml by adding distilled water.

Add 5ml of alkaline copper reagent to all the test tubes. Vortex and Incubate for 10 minutes at room temperature.

The solution in all the test tubes has turned blue in colour.

After incubation, add 600ul of Folin’s Ciocalteau reagent to all test tubes using micropipette. Vortex and Incubate for 20 minutes at room temperature.

After incubation, the color intensity varies accordingly with the concentration of protein present in the tubes.

Now record the absorbance of each solution at 650 nm using a colorimeter.

Plot the absorbance against amount of protein in milligrams to get a standard calibration curve. Check the absorbance of unknown sample and determine the concentration of the unknown sample from the standard curve plotted.

10. CHARACTERIZATION OF L-ASPARAGINASE [46-49]:

After fermentation process L-asparaginase were separated out by using filtration, centrifugation, and ammonium sulfate salt precipitation , this partially purified enzyme were used for characterization studies.

Effect of temperature on enzyme activity:

The enzyme was assayed in the reaction mixture containing 2.0ml of 0.5% casein solution in 0.1M carbonate buffer (pH 9.3) and 0.1ml of enzyme solution in the total volume of 2.1 ml. After incubation at $5^{O}C$, $37^{O}C$, $55^{O}C$ and $80^{O}C$ for 5mins, the reaction was stopped by adding 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 min. The reading was taken at 660 nm in UV- Spectrophotometer to monitor the enzyme activity.

Effect of pH on enzyme activity:

The above method was repeated using 0.1M carbonate buffer of different pH (2, 4, 6, 8 and10) and the enzyme activity was monitored spectrophotometrically.

11. CONFIRMATION OF ENZYME PRODUCTION BY SDS-PAGE [50-52]:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 3-mm slab gel of 6% acrylamide in a Tris-borate buffer pH 7.1 containing 0.1% SDS. The gels were stained with 0.025 Coomassie brilliant blue R-250 and destained.

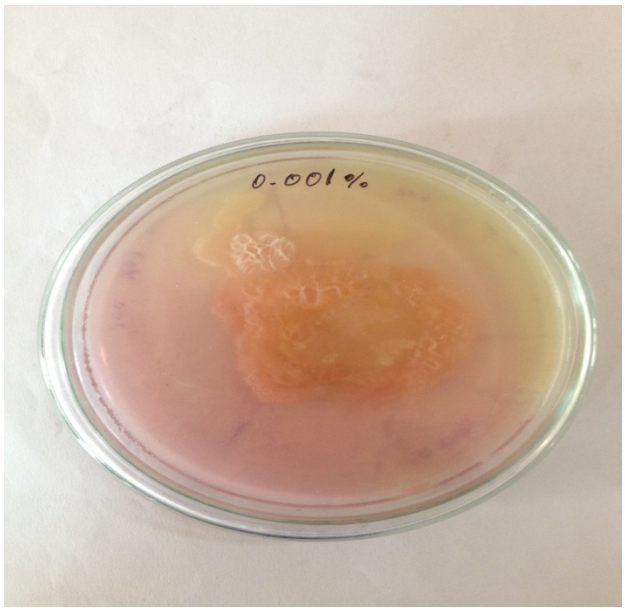
Protocol

1. Prepare polyacrylamide gel according to standard protocol.
2. Load samples and run gel at 50 mA (2 gels run in 1x SDS Running Buffer.
3. At this point, the gel stained with Coomassie blue (see below).
4. Place gel in a plastic container. Cover with isopropanol fixing solution and shake at room temperature. For 0.75 mm-thick gels shake 10 to 15 min; for 1.5 mmthick gels, shake 30 to 60 min.
5. Pour off fixing solution. Cover with Coomassie blue staining solution and shake at RT for 2 hr.
6. Pour off staining solution. Wash gel with 10% acetic acid to destain, shaking at RT.

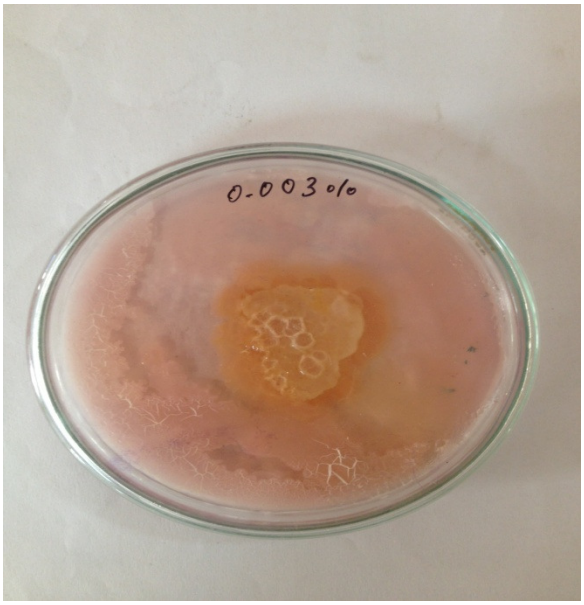
Almost all analytical electrophoresis of proteins were carried out under conditions that ensure dissociation of the proteins into their peptide subunits that minimize aggregation. The purified enzyme was resolved in SDS-PAGE by discontinuous buffer systems. The molecular weight of purified product of L-asparaginase was determined by 10% SDS-PAGE (3.2 ml of 30%Acrylamide, 2.08 ml of 1.5 M Tris (pH 8.8), 0.8 ml of 10% SDS, and 0.08 ml of 10% Ammonium per sulphate, 0.003 ml of TEMED and 2.56 ml of water). Later the gel was taken out carefully and stained with Coomassive brilliant blue until the bands were visible clearly identified by Staining and De-staining method.

RESULTS AND DISCUSSIONS

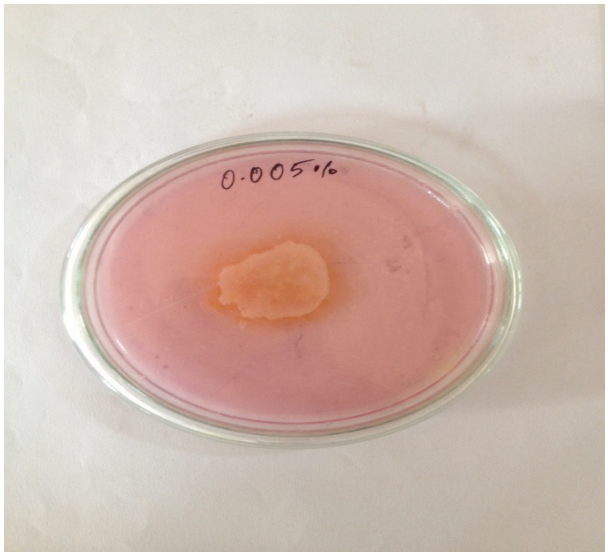
1. SCREENING THE CULTURE FOR L-ASPARAGINASE BY RAPID PLATE METHOD:



Phenol red =0.001%



Phenol red =0.003%



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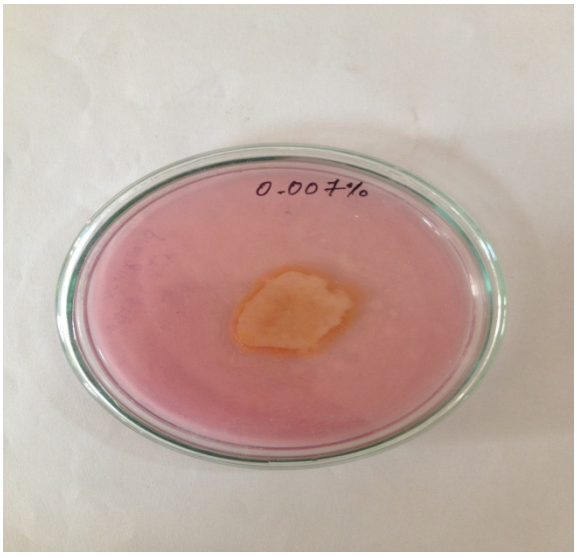
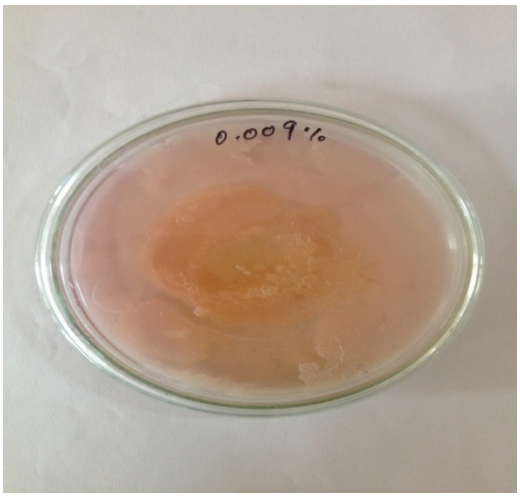


Phenol red =0.007%



Phenol red =0.009%

Plates: 1

Phenol red dye concentration range from 0.001% to 0.009%

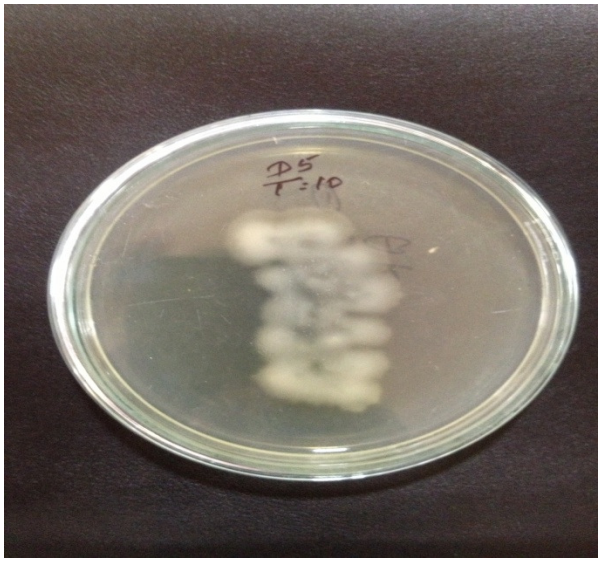
Zone diameter of screened strain:

Table: 8

SI no	Phenol red dye Concentration	Zone diameter of screened strain
1	0.001%	30mm
2	0.003%	29mm
3	0.005%	28mm
4	0.007%	26mm
5	0.009%	22mm

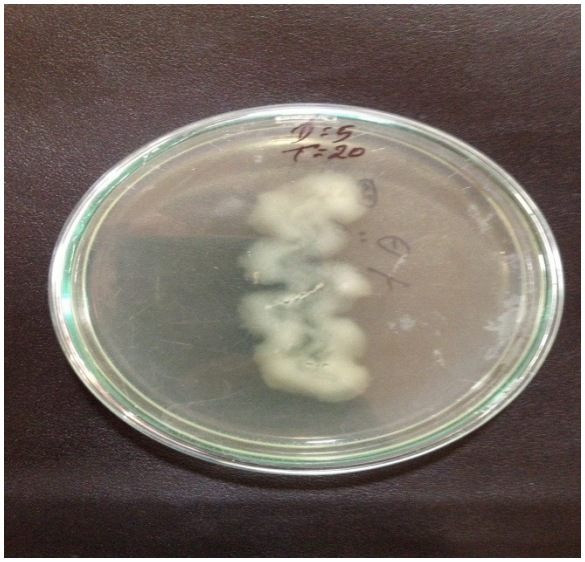
The rapid plate assay using phenol red dye revealed that presence of dye in medium did not inhibit the growth of the microorganism, Pink colored zone formed around the colony and size of the colony indicating that the strain capable of producing L-asparaginase .the method was quick and enzyme production can be observe from the plates as per Gulati *et al* method (1997).

2. IRRADIATION OF THE STRAIN BY UV IRRADIATION:



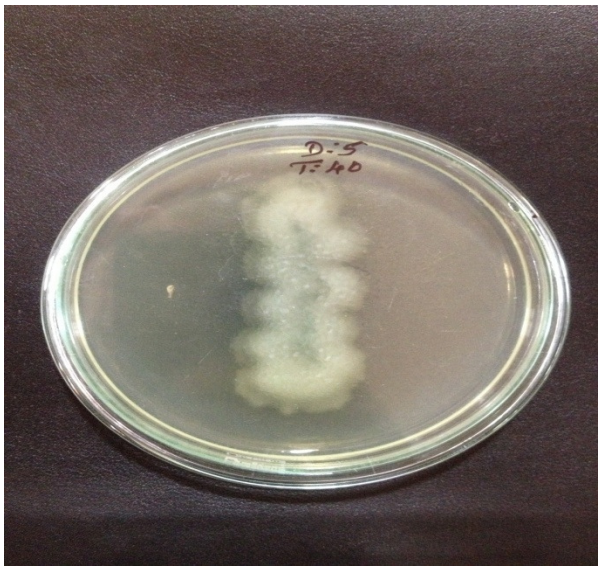
Distance=5cm

Time =10min



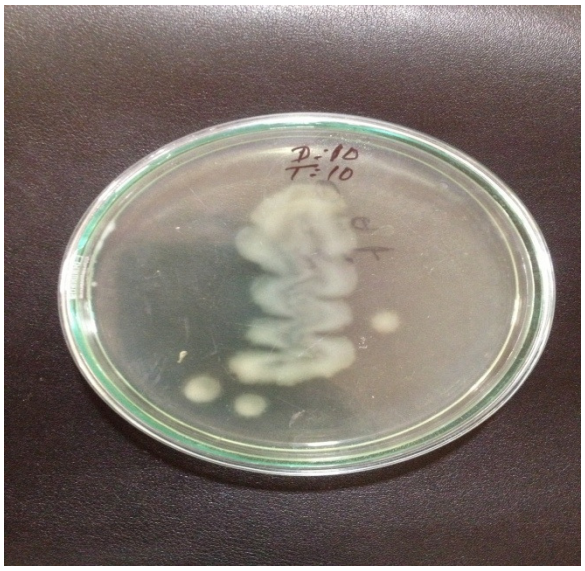
Distance=5cm

Time =20min



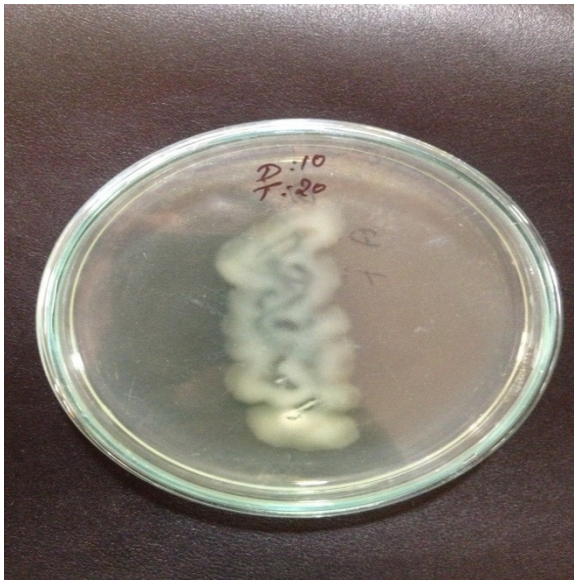
Distance=5cm

Time =40min



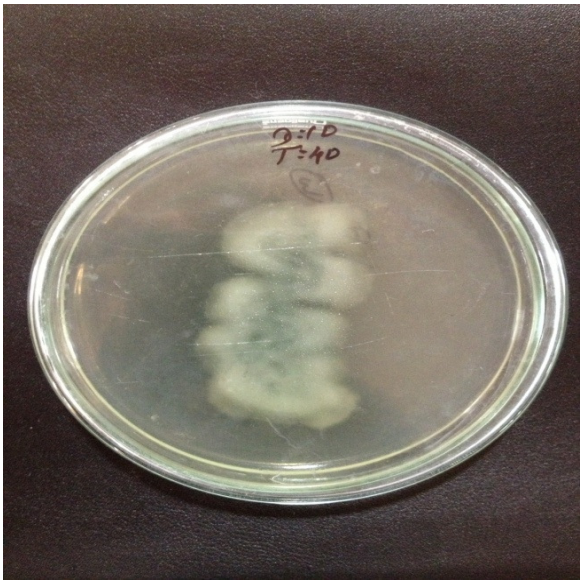
Distance=10cm

Time =10min



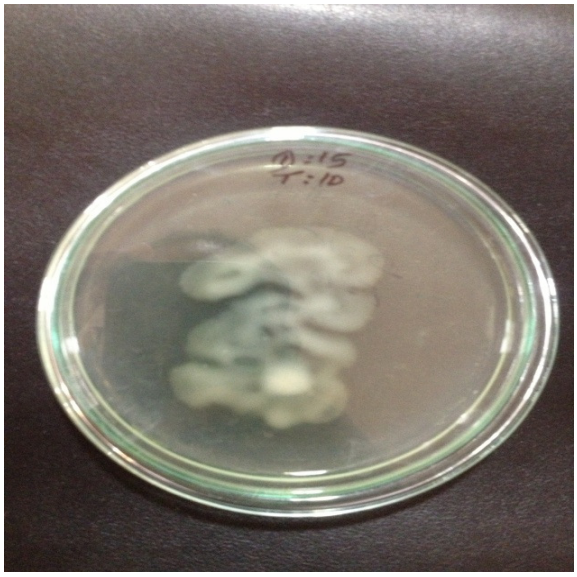
Distance=10cm

Time =20min



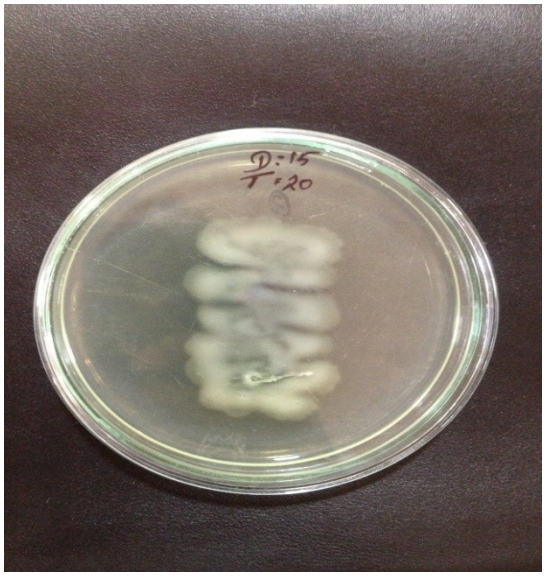
Distance=10cm

Time =40min



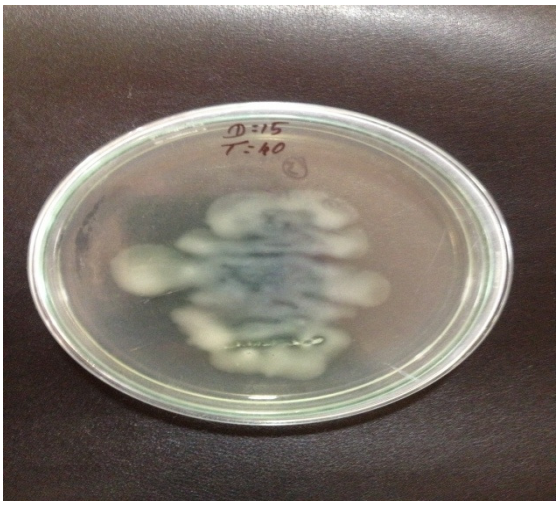
Distance=15cm

Time =10min



Distance=15cm

Time =20min



Distance = 15cm

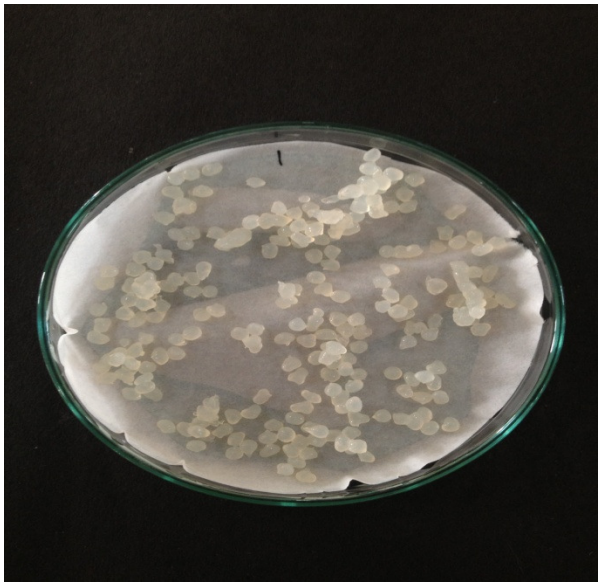
Time = 40min

Plates: 2

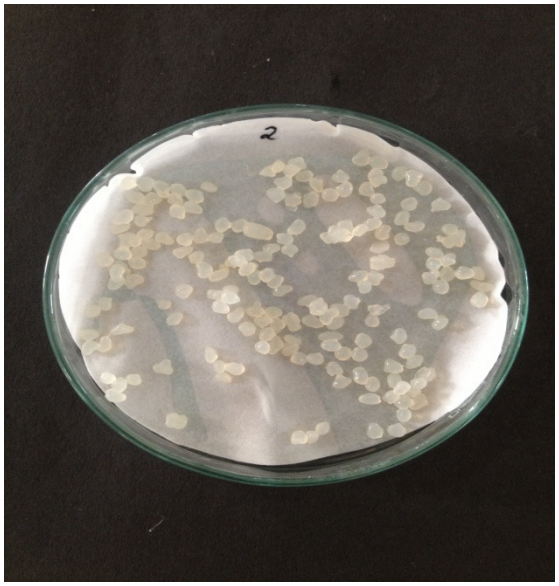
Distance = the plate distance from UV lamp Time = time of exposure to UV irradiation

The strain of *Pseudomonas aeruginosa* was exposed to UV irradiation from Philips lamp to improve the productivity of the strain from different distance (5, 10 and 15cm) and for different time (10, 20 and 40minutes) as per Amena *et al (2012).*

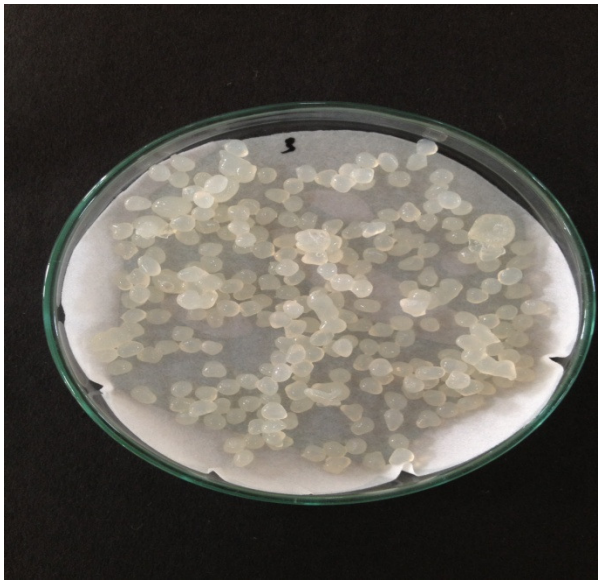
3. IMMOBILIZATION OF THE WHOLE-CELLS:



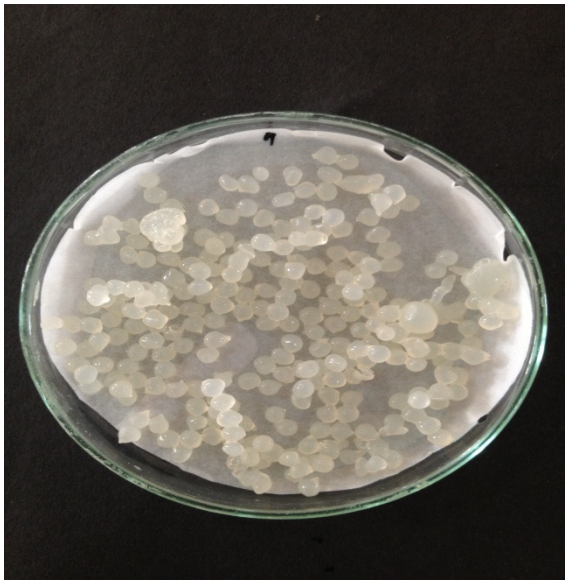
ir1



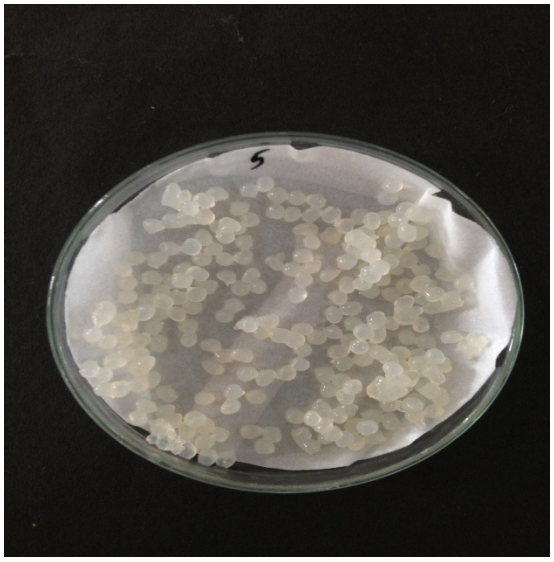
ir2



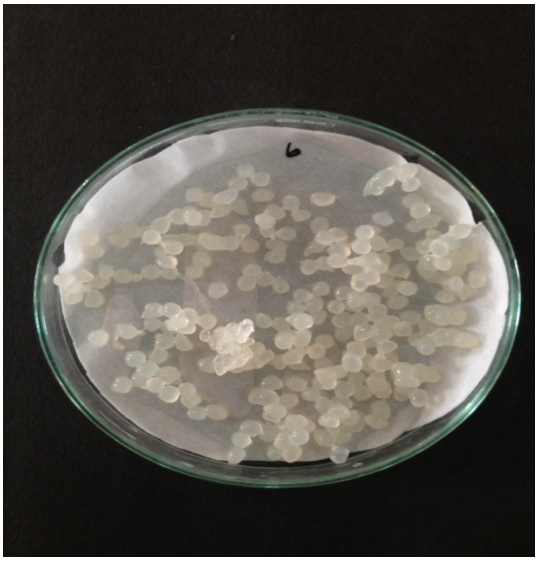
ir3



ir4

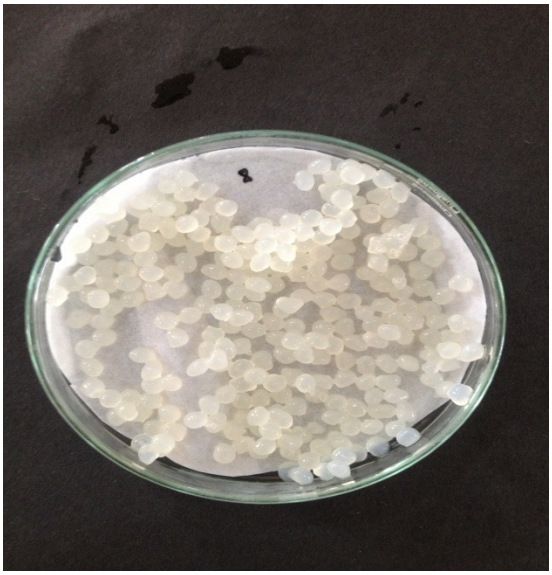


ir5

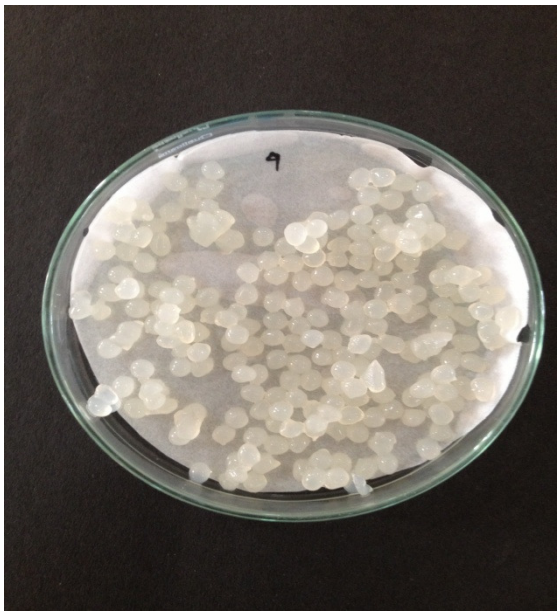


ir6

ir7



ir8



ir9

wild strain

Plates: 3

The whole cells of the mutated and the wild strains of Pseudomonas aeruginosa were immobilized by entrapment immobilization using sodium alginate as supporting material after cells were entrapped in the gel they left in the calcium chloride solution in the cool condition to cure then beads were removed from the solution and ready for use as per Deepali bisht *et al.*

4. ASSAY OF L-ASPARAGINASE FROM CRUDE EXTRACTS:

Table: 9

SI no	Strain	ENZYME ACTIVITY IU\ml		
		Day 3	Day 4	Day 5
1	Wild	10.83	31.66	16.66
2	ir1	32.5	21.66	18.33
3	ir2	21.66	25	22.5
4	ir3	29.16	32.5	31.66
5	ir4	29.16	36.66	37.5
6	ir5	42.5	**49.16**	43.33
7	ir6	38.33	45.83	41.66
8	ir7	30.83	29.16	29.16
9	ir8	26.66	33.33	22.5
10	ir9	27.5	28.33	25

C = wild strain

***ir* 1-9** = Irradiated strain

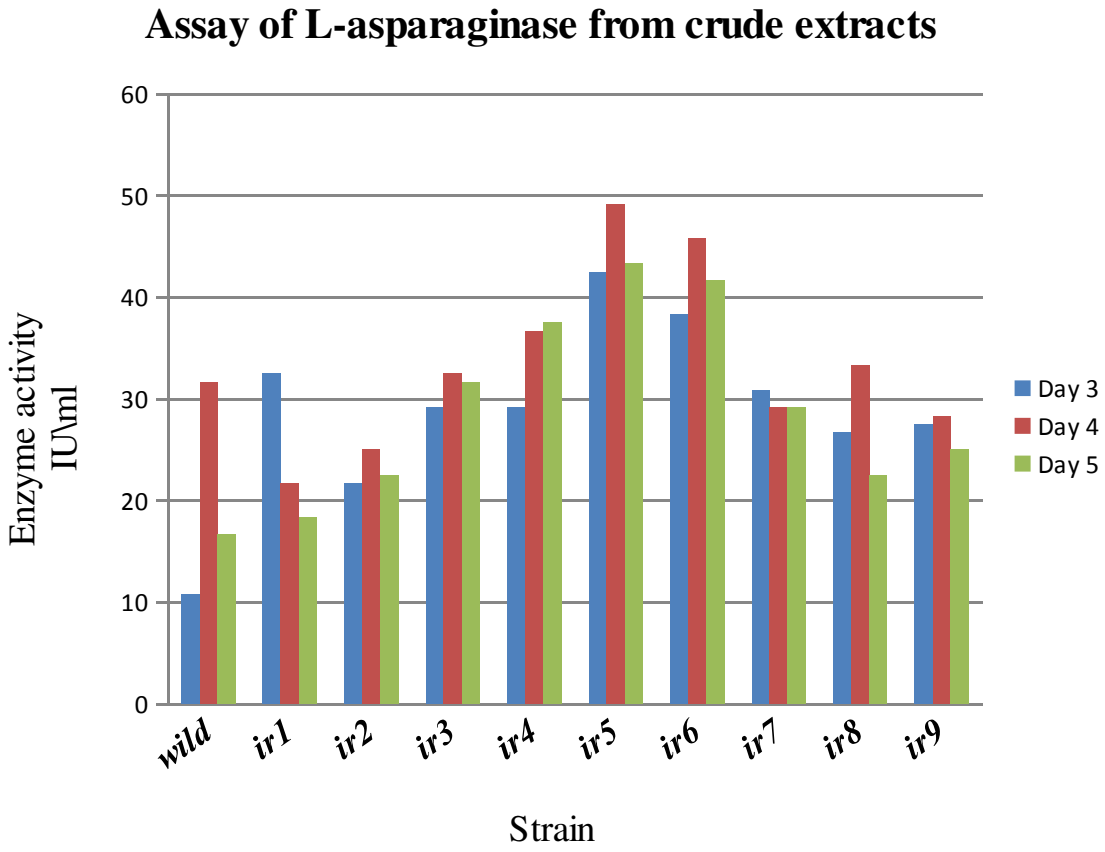

Assay of L-asparaginase from crude extracts
Enzyme activity IU\ml
60
50
40
30
20
10
0
wild
ir1
ir2
ir3
ir4
ir5
ir6
ir7
ir8
ir9
Strain
Day 3
Day 4
Day 5

Chart: 1

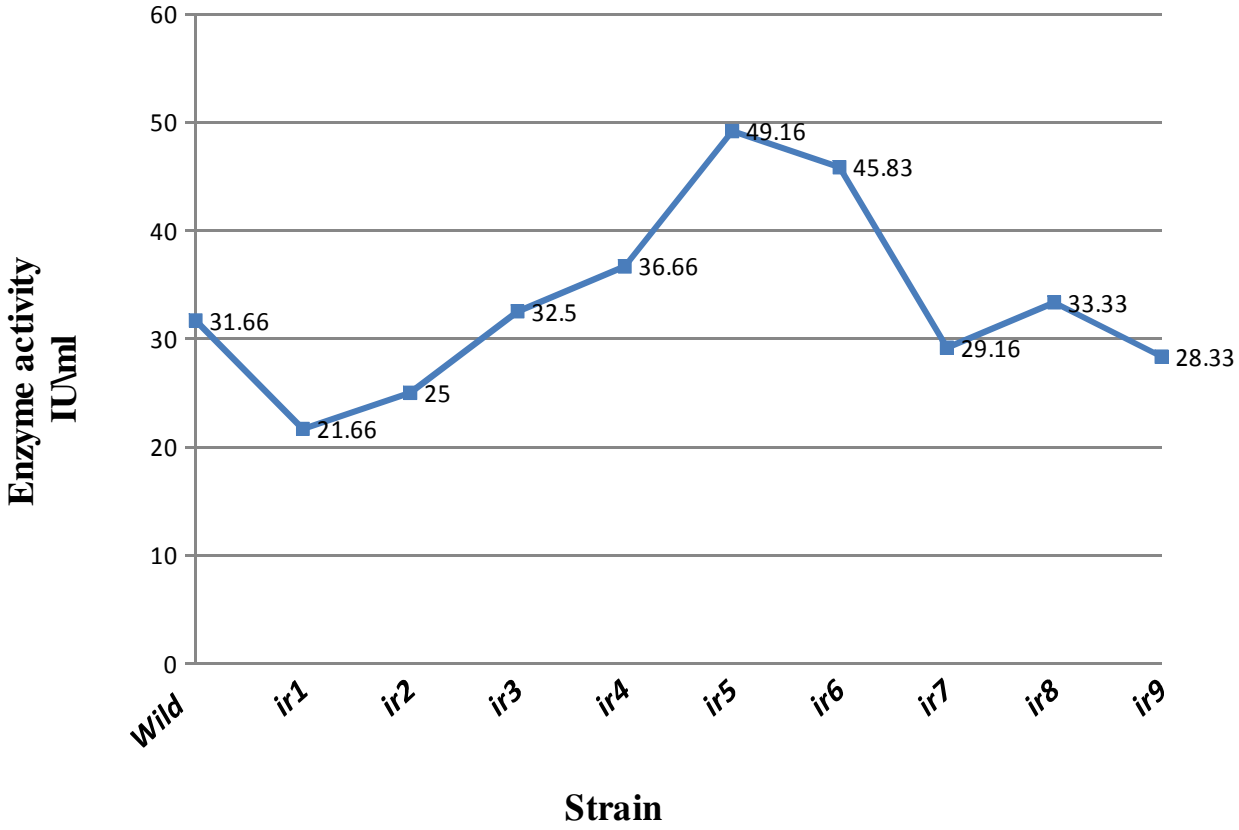


Chart: 2

The assay of crude enzyme at 96 hours (4days) of fermentation showed that mu5 has the highest yield 49.16 IU\ml of L-asparaginase compare to wild strain which yield 31.66 IU\ml of L-asparaginase. Hence the present study clearly indicates that L-asparaginase production was enhanced by 1.55 fold by mutation and immobilization.

5. ESTIMATION OF L-ASPARAGINASE BY LOWERY METHOD:

SI no	**Sample**	Absorbance(OD)at 650nm
1	Test	0.4
2	Standard 1	0.55
3	Standard 2	0.93
4	Standard 3	1.31
5	Standard 4	1.65
6	Standard 5	1.78

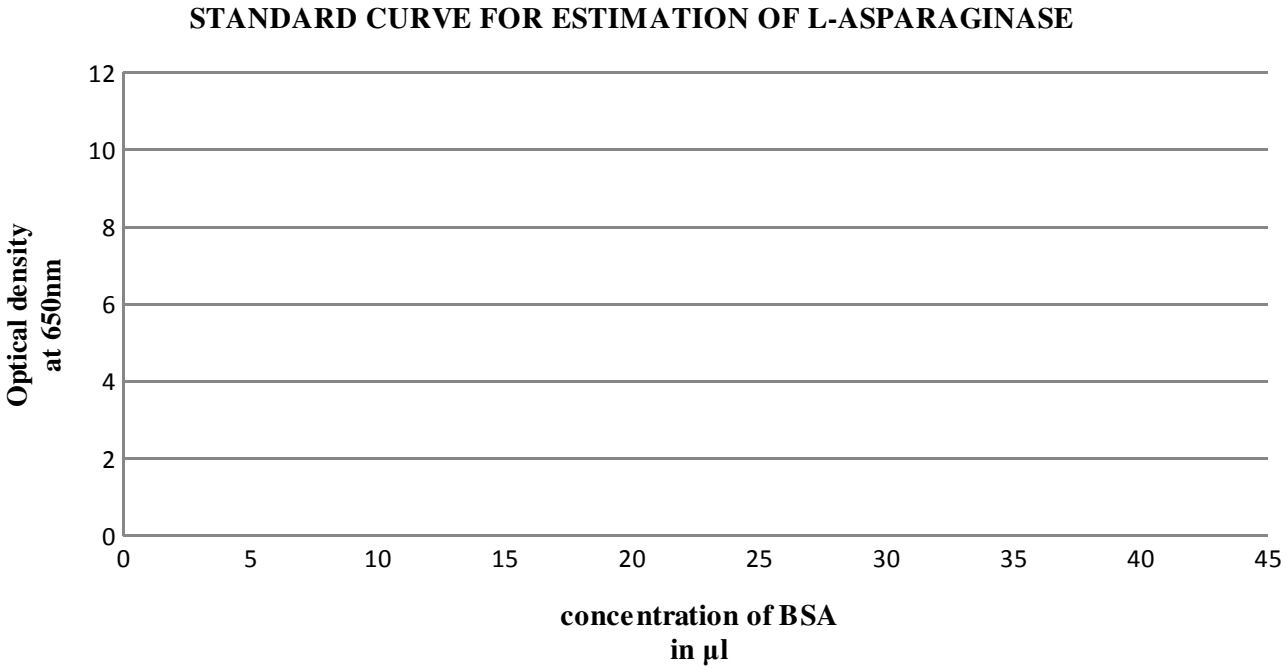


Chart: 3

L-asparaginase has been estimated by lowery method to determine the concentration of L-asparaginase in product by *Pseudomonas aeruginosa*. The concentration of L-asparaginase was found to be 750µg/ml.

6. CHARACTERIZATION OF L-ASPARAGINASE:

a) Effect of temperature on enzyme activity:

Table: 10

SI no	Temperature in ^{0}C	Enzyme activity
1	5 ^{0}C	29.16
2	37^{0}C	115
3	55^{0}C	40.83
4	80^{0}C	24.16

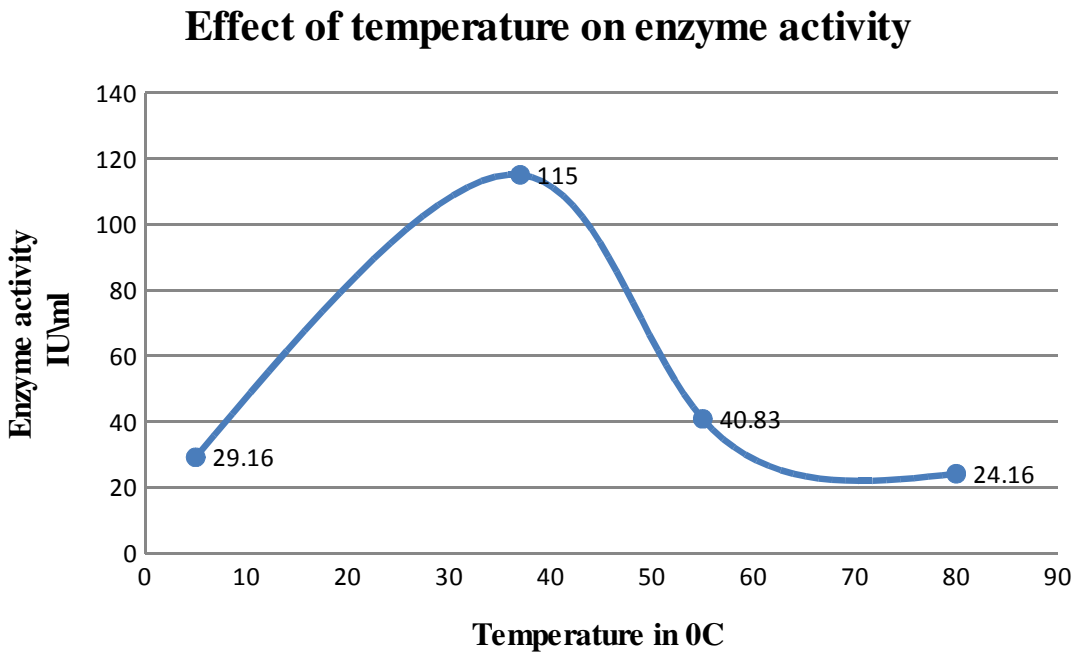


Chart: 4

A temperature profile of L-asparaginase showed that the enzyme had maximum activity at 37^{0}C Similar results were recorded for L- asparaginase from *Pseudomonas stutzeri* MB- 405 (Manna *et al*., 1995), *E. carotovora* (Maladkar *et al*., 1993), and *Staphylococcus* (Sobis & Mikucki, 1991). Also, Qian *et al.*, (1996)

b) Effect of pH on enzyme activity:

Table: 11

SI no	pH	Enzyme activity
1	3	20.83
2	5	70
3	7	183.33
4	9	227.5
5	11	154.16
6	13	110

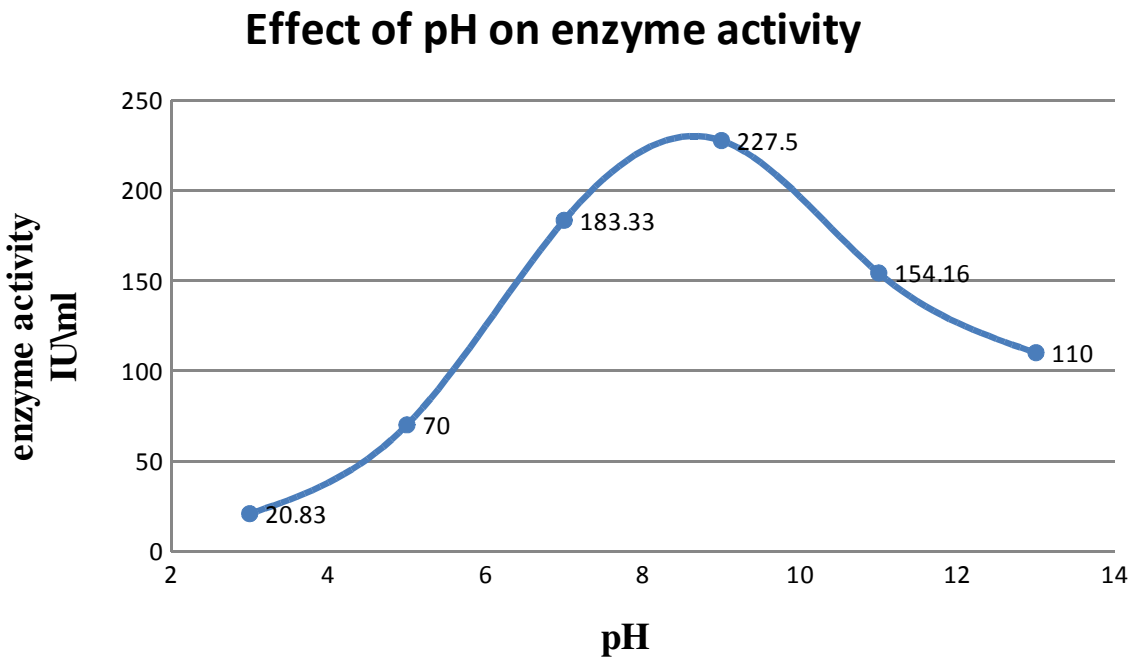


Chart: 5

The maximum L-asparaginase activity occurred when it was incubated with an optimum substrate concentration at pH 9. A similar pH value was obtained for *E. coli* (Castaman & Rodeghiero, 1993 and Liboshi *et al*., 1999)

7. CONFIRMATION OF ENZYME PRODUCTION BY SDS-PAGE:

Molecular characterization of L-asparaginase:

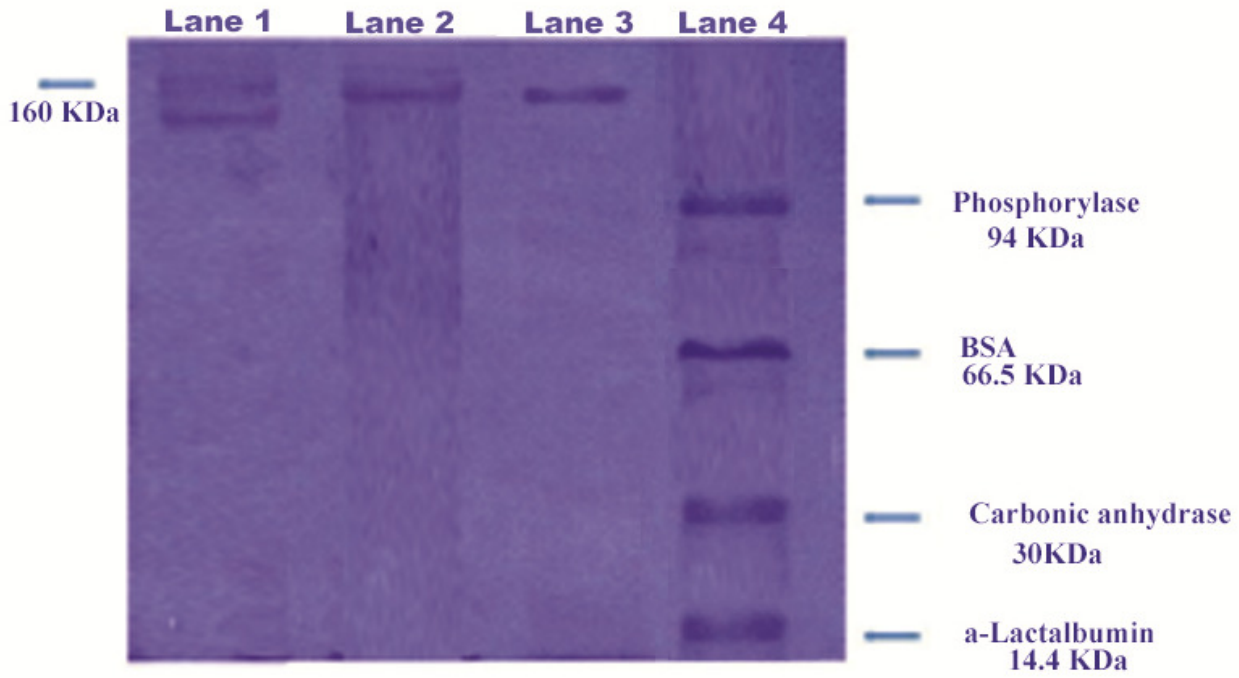


Figure: 2

Lane 1: Crude extracts (*P.aeruginosa*).

Lane 2: Ammonium sulphate precipitation.

Lane 3: Phosphate buffer treated.

Lane 4: Molecular Markers.

The partial purification of the L-asparaginase crude extract was loaded on 12% SDS-PAGE.the gel was stained with 0.025 Coomassive brilliant blue R-25 and destained. The following standard proteins were used for the molecular weight determination under identical condition .M w*t* of standard markers Phosphorylase 94 KDa, BSA 66.5 KDa, Carbonic anhydrase 30 KDa, α-lactalbumin 14.4KDa.

SDS-PAGE showed that the enzyme is one band by that was apparent molecular weight of *Pseudomonas aeruginosa* L-asparaginase was 160 KDa.

SUMMARY AND CONCLUSION

The discovery of the fact that L-Asparaginase is responsible for the action of the guinea-pig serum against the acute lymphoblastic leukemia has set a milestone in the field of medicine. After this discovery detailed information about the enzyme has been dug out. It has been proved that L-Asparaginase from *Escherichia coli* and *Erwinia carotovora* has anti-neoplastic activity against cancer and is being used as anti-cancer drug.

Microbial L-Asparaginase has been widely used as a therapeutic agent in the treatment of certain human cancers. With the development of its new functions a great demand for L-asparaginase is expected in the coming years [37].

The Bacterial strain of *Pseudomonas aeruginosa* was collected from MTCC Chandigarh.

The strain was screened for L-asparaginase production using (Gulati *et al*., 1997) method in which modified M9 medium was used. At 0.005% the higher productivity of L-asparaginase was shown by the formation of the biggest pink zone around colonies.

The strain has been irradiated by Physical method (UV irradiation), the irradiation of the strain was done by exposing the strain to UV irradiation of Philips lamp from different distances (5, 10 and 15cm) for different times (10, 20 and 40minutes) to get irradiated strain (*ir1- ir9).*

The culture strain has been immobilized by gel entrapment technique using sodium alginate as supporting material and preserved in Tris-HCl buffer (pH-8.0) at 4 °C until use.

Submerged fermentation was carried out by modified minimal fermentation broth that contained an amount of Soybean Casein Digest Medium broth. Then contained in a 250 ml Erlenmeyer flask was inoculated separately with the mutants of *Pseudomonas aeruginosa* and

incubated at 37 ºC in a shaker at 200 rev/min for 3-6days. At the end of the fermentation period, the crude enzyme was prepared by centrifugation at 10000 rpm for 20 min. The cell-free supernatant was taken as the crude enzyme.

Assay of enzyme was carried out as per Imad et al (1973) the enzyme activity was shown that at the highest productivity of L-asparaginase which 49.16 IU\ml was achieved by *ir5* compare with 31.66 IU\ml achieved by wild strain. Hence the present study clearly indicates that L-asparaginase production was enhanced by 1.55 fold by mutation.

The L-asparaginase was isolated from fermentation media as per El-Bessoumy *et al* the cell free supernatant was used as the crude enzyme preparation.

The purification was carried out at 4ºC on the crude extract. according to the modified method of Distasio *et al*. (1976) finely powdered ammonium sulfate was added to 80% saturation. The mixture was left for 12 h at 4ºC, followed by centrifugation at 8,000 rpm for 20 min at 4ºC. The precipitate was dissolved in a 0.01 M phosphate buffer pH 8.5.

L-asparaginase has been estimated by lowery method to determine the concentration of L-asparaginase in product by *Pseudomonas aeruginosa*. The concentration of L-asparaginase was found to be 1500µg/ml.

Studies of enzyme characterization by temperature and pH effect on enzyme activity showed that the highest enzyme activity 115 IU\ml was shown at 37^0C of temperature and enzyme activity of 227.5 IU was shown at pH 9.0.

The final product was examined for molecular weight using SDS-PAGE. the result showed that the M w*t* of L-asparaginase was 160 kDa when compared with standard molecular weight protein marker. To conclude that this study showed by mutation of the strain by UV irradiation, Immobilization of the whole cell and optimization of the basic fermentation media the production of L-asparaginase from *Pseudomonas aeruginosa* was increased up to 1.55 folds that gives hope to increase the productivity of L-asparaginase which will meet the demand of patients around the world.

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